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(54) Title: MYELIN BASIC PROTEIN PEPTIDES AND USES THEREOF

(57) Abstract

This invention pertains generally to the treatment of autoimmune diseases of the central nervous system characterized by demyelination. In particular, this invention pertains to novel peptides derived from human myelin basic protein (MBP). When complexed with the appropriate major histocompatibility complex (MHC) molecule, these peptides can be used to treat multiple sclerosis and other demyelinating autoimmune diseases.

MYELIN BASIC PROTEIN PEPTIDES AND USES THEREOF

FIELD OF THE INVENTION

This invention pertains generally to the treatment of autoimmune diseases of the central nervous system characterized by demyelination. In particular, this invention pertains to novel peptides derived from human myelin basic protein. These peptides can be used as pharmaceuticals to treat multiple sclerosis and other demyelinating autoimmune diseases alone or complexed with the appropriate major histocompatibility complex (MHC) molecule.

BACKGROUND OF THE INVENTION

Multiple sclerosis (MS) is a chronic, inflammatory, autoimmune disease of the human central nervous system (CNS) characterized by demyelination and by local infiltrates of macrophages, plasma cells and T cells in the CNS (Allen (1991) *Pathology of Multiple Sclerosis*, p341 in *McAlpine's Multiple Sclerosis*; Matthewse, *et al.*, eds, Churchill Livingstone, Edinburgh). MS is an autoimmune disease directed at myelin and the myelin-producing oligodendrocyte. In MS and other demylelinating diseases, T cell clones specific for a component of myelin, myelin basic protein (MBP), cause the demyelination of nerve sheaths in the CNS. In addition to MBP-restricted T lymphocyte clones, demyelinating inflammatory lesions can contain multiple nonrestricted immune cells capable of mediating tissue injury (Zamvil (1990) *Ann. Rev. Immunol.* 8:579-621; Wucherpfenning (1991) *Immunol. Today* 12:277-282; Martin (1992) *J. Immunol.* 148:1359-1366; Martin (1992) *Ann. Rev. Immunol.* 10:153-187).

Major histocompatibility complex (MHC) class II (DR) molecules are heterodimers displayed on the cell surface of antigen processing/ presenting cells (APCs). In demyelinating disease, APC Class II molecules "present" MBP self-peptides derived from myelin and oligodendrocytes to helper (CD4+) T lymphocytes. The Class II molecules literally present the peptide to the T cell by binding the peptide in a "peptide binding site" or

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groove which is structurally positioned at the end of the molecule farthest from the cell membrane. The peptide, or antigen, binding site, is also called the "antigen binding pocket" or "MHC groove." (Brown (1993) *Nature* 364:33-39; Stern (1994) *Structure* 2:245-251).

The binding of a peptide to a peptide binding site is dependent on the primary amino acid sequence of both the peptide and the Class II molecule. Only high affinity binding between a peptide and a Class II polypeptide will form a complex which will be presented extracellularly to T cells. The affinity, or strength, of this intermolecular attraction is determined by the same factors that exist for all peptide:polypeptide binding reactions, *e.g.*, conformation, hydrogen bonding, charge, ionic interactions. The limitations created by primary sequence are further limited by a size limitation; the peptide binding site is believed to be able to accomodate peptides ranging from about eight to about twenty amino acids in length (the "agretope" is that portion of the peptide recognized by the MHC molecule). As a result, different portions of an antigenic polypeptide are typically presented by different Class II molecules. Thus, when a peptide is internalized by an APC and proteolytically processed into fragments, only a single or small number of peptides will bind with high affinity, *i.e.*, be "specifically bound," to a specific Class II molecule. As only a limited number of Class II molecules are expressed in an individual, only a limited repetoire of peptides from a polypeptide antigen are presented.

For example, in the case of MS, a single region of MBP is involved in binding a limited set of Class II molecules to help generate anti-myelin autoreactive T cell clones. In some MS patients, a putative pathognomonic "immunodominant" MBP peptide and the Class II molecules to which it binds are suggested to be the MBP peptide spanning residues 83 to 102 (MBP 83-102) which binds to either the Class II DR allele designated DRB1*1501 and or to DRB5*0101 (other Class II alleles and MBP peptides may also be mediating MS or other demyelinating diseases in different individuals).

Recognition of the peptide:Class II complex by a helper CD4+ T lymphocyte is mediated by the binding of the T cell's clonally specific receptor (TCR) to the complex. The TCR's affinity to the complex is determined by its attraction to both the Class II molecule and the peptide occupying the antigen binding pocket (the portion of the peptide recognized by TCR is called the "epitope"). High affinity TCR binding to the APC complex activates the T cell, inducing its clonal proliferation and secretion of immunomodulatory cytokines (which

can be stimulatory or immunosuppressive). Thus, there is an amplification of an immune response specific for the antigenic peptide (the epitope) that is displayed by the Class II polypeptide. If the peptide is a self peptide, the response can be an autoimmune reaction.

Interfering with the ability of peptide:Class II complex binding to TCR can inhibit the development of or suppress an autoimmune reaction. For example, administration of antibodies to MHC Class II polypeptides can interfere with complex:TCR binding and the resultant pathogenic immune reaction. Antibodies to autoreactive TCR or T cell clones can have the same effect.

An alternative immunosuppressive strategy can be used to exploit the need for a co-stimulatory signal to the T cell by the APC. To activate CD4+ T cells, TCR binding by Class II:peptide is not sufficient. An additional, "co-stimulatory" signal is needed (not an MHC molecule). Typically, the required co-stimulatory signal is provided by an APC cell surface protein. Significantly, interaction of Class II:peptide with TCR without APC co-stimulation not only does not induce T cell activation, but it also results in a state of antigen-specific unresponsiveness on rechallenge, known as anergy *in vitro* and tolerance *in vivo* (Boussiotis (1994) *Curr. Opin. Immunol.* 6:797-807; Park (1997) *Eur. J. Immunol.* 27:1082-1090). This suppression and re-challenge non-responsiveness has been hypothesized to be by T cell clonal anergy or by a nonresponsiveness induced by immunosuppressive cytokines (Schwartz (1989) *Cell* 57:1073-1081; Quill (1987) *J. Immunol.* 138:3704-3712).

While the twenty amino acid long MBP 83-102 may be the immunodominant, naturally processed MBP peptide in some individuals, synthetic or recombinant modifications of myelin or peptide MBP 83-102 which bind to Class II molecule and/or T cell receptor with greater affinity can be therapeutically efficacious. Such peptides would be better reagents to inhibit the pathogenic autoimmune reaction, *e.g.*, through their administration as tolerance-inducing soluble Class II:peptide complexes. By providing novel MBP peptides capable of binding to Class II molecules and TCRs, the present invention fulfills these and other needs.

Current treatment for autoimmune disease consists primarily of treating the symptoms, but not intervening in the etiology of the disease. Broad spectrum immunosuppressive agents which have numerous undesirable side effects are typically employed. The inadequate treatments presently available illustrate the urgent need to identify new agents that prevent or suppress MHC-restricted immune responses, but avoid undesirable

side effects, such as nonspecific suppression of an individual's overall immune response. Compounds capable of selectively suppressing autoimmune responses at the helper CD4+ T cell level would provide a safer, more effective treatment. By providing novel MBP peptides and peptide:Class II molecule complexes, the present invention fulfills these and other needs.

SUMMARY OF THE INVENTION

The invention provides an isolated myelin basic protein (MBP) peptide, said peptide characterized by having an amino acid sequence Phe -X - Lys - Asn - Ile - Val - X - X - X - Thr - X -X, wherein X is any amino acid. In one embodiment, the isolated myelin basic protein peptide is characterized by having an amino acid sequence Phe - Phe - Lys - Asn - Ile - Val - Thr - Pro - Arg - Thr - Pro - Pro, or a conservative substitution thereof. The isolated myelin basic protein peptide can be linked to a heterologous sequence. In one embodiment, linkage of the myelin basic protein peptide to a heterologous sequence results in a fusion protein.

The invention also provides an isolated myelin basic protein peptide which specifically binds to an antibody directed against a peptide characterized by having an amino acid sequence Phe - Phe - Lys - Asn - Ile - Val - Thr - Pro - Arg - Thr - Pro - Pro.

In another embodiment, the invention provides an isolated nucleic acid encoding a myelin basic protein peptide, said peptide characterized by having an amino acid sequence Phe - X - Lys - Asn - Ile - Val - X - X - X - Thr - X - X , wherein X is any amino acid. The encoded myelin basic protein peptide can be characterized by having an amino acid sequence Phe - Phe - Lys - Asn - Ile - Val - Thr - Pro - Arg - Thr - Pro - Pro , or a conservative substitution thereof. In one embodiment, the isolated nucleic acid comprises SEQ ID NO:1.

The invention also provides a composition comprising an MHC Class II complex capable of binding a T cell receptor, the complex consisting essentially of: an MHC Class II polypeptide comprising an extracellular domain of an MHC Class II molecule sufficient to form an antigen binding pocket, said component being encoded by an allele associated with an autoimmune disease directed to myelin basic protein, wherein the Class II component is soluble under physiological conditions in the absence of detergent or lipid; and, a myelin basic protein having an amino acid sequence Phe -X - Lys - R_1 - Ile - Val - X -

X - X - Thr - X -X , wherein X is any amino acid, and R_1 is Asn or Gln; wherein the myelin basic protein peptide is bound to the MHC Class II component antigen binding pocket. The myelin basic protein peptide can have an amino acid sequence Phe - Phe - Lys - Asn - Ile - Val - Thr - Pro - Arg - Thr - Pro - Pro, or a conservative substitution thereof. The composition can comprise a fusion protein and/or an effector composition. In alternative embodiments, the autoimmune disease is multiple sclerosis, or the class II polypeptide comprises the antigen binding pocket of an HLA DR2.

The invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a pharmacologically effective amount of a peptide characterized by having an amino acid sequence Phe -X - Lys - Asn - Ile - Val - X - X - X - Thr - X -X, wherein X is any amino acid.

The invention also provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a pharmacologically effective amount of a composition comprising an MHC class II complex capable of binding a T cell receptor, the complex consisting essentially of: an MHC class II polypeptide comprising an extracellular domain of an MHC class II molecule sufficient to form an antigen binding pocket, said component being encoded by an allele associated with an autoimmune disease directed to myelin basic protein, wherein the class II component is soluble under physiological conditions in the absence of detergent or lipid; and, a myelin basic protein having an amino acid sequence Phe -X - Lys - R_1 - Ile - Val - X - X - X - Thr - X -X , wherein X is any amino acid, and R_1 is Asn or Gln; wherein the myelin basic protein peptide is bound to the MHC class II component antigen binding pocket.

The invention provides an antibody, specifically immunoreactive under immunologically reactive conditions, to a myelin basic protein peptide, said peptide characterized by having an amino acid sequence Phe - Phe - Lys - Asn - Ile - Val - Thr - Pro - Arg - Thr - Pro - Pro. In another embodiment, the invention provides an antibody is specifically immunoreactive under immunologically reactive conditions, to a myelin basic protein peptide comprising the peptide encoded by a nucleic acid of encoding a myelin basic protein peptide, said peptide characterized by having an amino acid sequence Phe - X - Lys - Asn - Ile - Val - X - X - X - Thr - X - X , wherein X is any amino acid.

The invention provides a method for inhibiting a T cell mediated immune response against myelin basic protein in a subject, comprising administering to the subject an isolated myelin basic protein (MBP) peptide, said peptide characterized by having an amino acid sequence Phe -X - Lys - Asn - Ile - Val - X - X - X - Thr - X -X, wherein X is any amino acid, in an amount effective to treat the T cell mediated immune response.

The invention provides a method for inhibiting a T cell mediated immune response against myelin basic protein in a subject, comprising administering to the subject a composition comprising an MHC Class II complex capable of binding a T cell receptor, the complex consisting essentially of: an MHC Class II polypeptide comprising an extracellular domain of an MHC Class II molecule sufficient to form an antigen binding pocket, said component being encoded by an allele associated with an autoimmune disease directed to myelin basic protein, wherein the Class II component is soluble under physiological conditions in the absence of detergent or lipid; and, a myelin basic protein having an amino acid sequence Phe -X - Lys - R_1 - Ile - Val - X - X - X - Thr - X -X , wherein X is any amino acid, and R_1 is Asn or Gln; wherein the myelin basic protein peptide is bound to the MHC Class II component antigen binding pocket.

The invention provides a method for inhibiting a T cell mediated immune response against myelin basic protein in a subject wherein the T cell mediated immune response causes a pathology to a neurological system. In one embodiment, the pathology to the neurological system is characterized as multiple sclerosis.

The invention provides a method of identifying a T cell epitope on an antigen which, when bound to the antigen binding pocket of an MHC Class II molecule, is capable of binding to a T cell receptor, such binding triggering an extracellular acidification reaction by a T cell expressing the T cell receptor, said method comprising the steps of: a) providing a composition comprising the T cell epitope bound to the antigen binding pocket of an MHC Class II molecule; b) contacting a T cell expressing the T cell receptor with the epitope; and, c) measuring the extracellular acidification, wherein a change in the extracellular acidification indicates the binding of T cell epitope to the T cell receptor. In one embodiment, the change in extracellular acidification is measured using a microphysiometer.

A further understanding of the nature and advantages of the present invention may be realized by reference to the remaining portions of the specification, the figures and claims.

All publications, patents and patent applications cited herein are hereby expressly incorporated by reference for all purposes.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A shows the MBP peptides used in the studies described in Example 1. Figure 1B shows MBP peptides with various alanine substituted residues used in the studies described in Example 1.

Figures 2A, 2B and 2C show the results of analyses described in Example 1, demonstrating increased levels of gamma-IFN, TNF-beta and extracellular acidification rates, respectively, with six N-terminal truncated MBP peptides. Figures 2D, 2E and 2F show results of analyses described in Example 1, comparing the gamma-IFN, TNF-beta and acidification rates, respectively, of five N-terminus truncated MBP peptides.

Figures 3A, 3B, and 3C, show the results of analyses described in Example 1, comparing the gamma-IFN, TNF-beta and acidification rates, respectively, induced by C-terminal truncated MBP peptides. Figures 3D, 3E, and 3F, show the results of analyses described in Example 1, comparing the gamma-IFN, TNF-beta and acidification rates, respectively, induced by C-terminal truncated MBP peptides.

Figures 4A, 4B and 4C show the results of analyses described in Example 1, demonstrating MBP peptide residues necessary to induce gamma-IFN, TNF-beta along with increased acidification rates, respectively, when presented to SS8T cells by Class II DRB5*0101. Figures 4D, 4E and 4F show the results of analyses described in Example 1, demonstrating MBP peptide residues necessary to induce gamma-IFN, TNF-beta along with increased acidification rates, respectively, when presented to SS8T cells by Class II DRB5*0101.

Figure 5A shows, in highlights (boxed area), the core TCR recognition sequence as MBP(91-100) (SEQ ID NO:38). Figure 5B shows (by arrows) the important MBP amino acid residues involved in TCR contact as F-91, K-93, N-94, I-95, and V-96.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to methods and compositions that can be used to inhibit those aspects of the immune system which are responsible for undesirable autoimmunity. This invention is a first description of a novel set of peptides derived from human myelin basic protein (MBP) that can be used in the treatment of autoimmune mediated demyelinating disease, in particular, multiple sclerosis (MS). The invention provides therapeutic compositions comprising novel MBP peptides for, *e.g.*, inducing oral or general tolerance. Also provided are a soluble MBP peptide:Class II molecule complex for targeting T cells mediating the autoimmune reaction. The complex targets T cell clones reactive to MBP by binding to autoreactive T cell receptor (TCR) capable of binding the MBP:Class II complex. The binding of the MBP:Class II complex to the T cell is therapeutically efficacious for inhibiting the development of or treating autoimmune demyelinating disease. While the invention is not limited by any particular mechanism of action, the compositions of the invention are pharmaceutically active by, *e.g.*, inducing T cell clonal anergy and tolerance or inducing a cytokine-mediated immunosuppressive immune response.

The invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a pharmacologically effective amount of a peptide characterized by having an amino acid sequence Phe -X - Lys - Asn - Ile - Val - X - X - X - Thr - X -X, wherein X is any amino acid. Administration of the peptide alone can be used to induce oral tolerance, which is a phenomenon of systemic, antigen specific, immunological hyporesponsiveness that results from oral administration of a protein. The mechanism by which oral tolerance is generated depends on the amount of antigen (peptide) administered. Low doses favor induction of regulatory immunosuppressive helper CD4+ T cells. Higher doses favor clonal deletion and anergy (tolerance). Regulatory T cells induced by low doses of oral antigen, when re-stimulated, secrete immunosuppressive cytokines that suppress the inflammatory response in an antigen nonspecific manner. Orally administered autoantigens have been shown to suppress a wide variety of experimental autoimmune diseases, including the most widely studied animal model for MS, the murine demyelinating disease experimental autoimmune encephalomyelitis (EAE). Murine MBP peptide has been used to generate an antigen-specific oral tolerance and to prevent development of acute EAE (Bitar (1988) *Cell. Immun.* 112:364-370; Higgins (1988) *J. of Immun.* 140:440-445; Khoury (1992) *J. Exp. Med.*

176:1355-1364; Whitacre (1996) *Clin. Immunol. Immunopathol.* 80:S31-S39). In one study, oral administration of bovine myelin to MS patients induced T cells that secreted the immunosuppressive cytokine TGF-beta-1 (Fukaura (1996) *J. Clin. Invest.* 98:70-77). Thus, the novel human MBP peptides of the invention can be efficacious in the generation of an antigen-specific immunosuppressive reaction, as in the induction of oral tolerance.

The present invention is also directed to purified complexes comprising at least an effective antigen-binding portion of a dimeric Class II molecule or a single chain (monomeric) subunit of a Class II and an MBP peptide of the invention. An "effective antigen-binding portion" is the mininum amount of dimeric or monomeric Class II molecule necessary to bind MBP peptide with sufficient affinity to create a complex capable of being recognized by and specifically bind to an autoreactive TCR. The complex of the invention, comprising MBP peptide and the appropriate Class II molecule, can be bound or otherwise associated together covalently or noncovalently. A third component, such as an "effector" component, can also be included in the complex of the invention. The effector component can be a cytotoxic agent, *i.e.*, a toxin, radioisotope, apoptotic-inducing agent, and the like, to selectively eliminate or neutralize the targeted, autoimmune T cell population.

In other aspects, the invention is directed to pharmaceutical compositions wherein the peptide and complexes of the invention are the active ingredients. These compositions are used to down-regulate or eliminate autoreactive components of the immune system and to treat autoreactive, demyelinating, T cell mediated immune responses. The invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a pharmacologically effective amount of the MBP peptide:Class II complex. The complexes selectively bind MBP autoreactive T cells. While the invention is not limited to any specific means by which the compositions can be pharmaceutically active, the peptides and complexes can induce clonal anergy/ tolerance or induce a cytokine-mediated immunosuppressive immune response. The complexes can be further conjugated to cytotoxic agents to specifically eliminate targeted autoreactive T cells.

I. Nucleic Acids Encoding MBP Peptides and MHC Class II DR Alleles and Recombinant Expression of these Nucleic Acids

This invention provides novel myelin basic protein (MBP) peptides and complexes of these MBP peptides and MHC Class II polypeptides comprising at least the extracellular domain of a Class II molecule sufficient to form an antigen binding pocket, and nucleic acid encoding these MBP peptides and Class II polypeptides. In addition to providing synthetic forms of MBP and Class II nucleic acids, the invention provides recombinantly generated nucleic acid, MBP peptides, and Class II polypeptides. As nucleic acid encoding these peptides and proteins can be expressed *in vitro* or *in vivo*, the invention provides for a variety of means of expressing these sequences, including expression cassettes, vectors, cell lines, transgenic plants and animals, and the like.

The invention can be practiced in conjunction with any method or protocol known in the art, which are well described in the scientific and patent literature. Therefore, only a few general techniques will be described prior to discussing exemplary methodologies and examples relative to the novel reagents and methods of the invention.

General Techniques

The MBP peptide- and MBP:Class II complex-encoding genes and nucleic acids of this invention, whether RNA, cDNA, genomic DNA, or hybrids thereof, can be isolated from a variety of sources; genetically engineered; expressed recombinantly; or synthesized *in vitro*. Nucleic acids encoding for the MBP and MBP:Class II complexes of the invention can be expressed in transgenic plants and animals, transformed cells and cell lines, in a transformed cell lysate, or in a partially purified or a substantially pure form. Techniques for nucleic acid manipulation of genes encoding the MBP and MBP:Class II complexes of the invention, such as site specific mutagenesis, generating libraries, subcloning into expression vectors, labeling probes, sequencing DNA, DNA hybridization are described in the scientific and patent literature, see *e.g.*, Sambrook, ed., MOLECULAR CLONING: A LABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989) ("Sambrook"); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel, ed. John Wiley & Sons, Inc., New York (1997) ("Ausubel"); and, LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY: HYBRIDIZATION WITH NUCLEIC ACID PROBES, *Part I. Theory and Nucleic Acid Preparation*, Tijssen, ed. Elsevier, N.Y. (1993) ("Tijssen"). Sequencing methods typically

use dideoxy sequencing (Sequenase, U.S. Biochemical), however, other kits and methods are available and well known to those of skill in the art.

Nucleic acids and proteins are detected, isolated and quantified in accordance with the teachings and methods of the invention described herein by any of a number of general means well known to those of skill in the art. These include, *e.g.*, analytical biochemical methods such as NMR, spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and hyperdiffusion chromatography, various immunological methods, such as fluid or gel precipitin reactions, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immuno-fluorescent assays, Southern analysis, Northern analysis, Dot-blot analysis, gel electrophoresis, RT-PCR, quantitative PCR, other nucleic acid or target or signal amplification methods, radiolabeling, scintillation counting, and affinity chromatography.

Mutations can be introduced into a nucleic acid by a variety of conventional techniques, well described in the scientific and patent literature. For example, one rapid method to perform site-directed mutagenesis efficiently is the overlap extension polymerase chain reaction (OE-PCR) (Urban (1997) *Nucleic Acids Res.* 25:2227-2228).

MHC Class II DR Alleles

The invention provides nucleic acids encoding novel myelin basic protein (MBP) peptides and MHC Class II polypeptides comprising at least the extracellular domain of an MHC Class II molecule sufficient to form an antigen binding pocket. The invention provides an example of human DR Class II alleles encoding polypeptide capable of binding the MBP peptides of the invention, specifically, the alleles designated DRB1*1501 and DRB5*0101 (see, e.g., Weber (1993) *Proc. Natl. Acad. Sci. 90:11049-11053*). However, the complexes of the invention are not limited to these DR alleles or their corresponding Class II polypeptides. The MBP:Class II complexes of the invention include any polypeptide comprising an MHC Class II antigen binding pocket, or functional equivalent thereof, which can bind an MBP peptide of the invention with sufficient affinity to be used in the methods of the invention. Means to identify known such DR alleles and Class II polypeptides are well known in the scientific and patent literature, *e.g.*, through electronic databanks, as Medline, GenBank, and the like. Means to identify MHC Class II DR alleles encoding polypeptide

having an antigen binding pocket capable of binding a particular peptide, such as, *e.g.*, an MBP peptide of the invention, are also well known in the scientific and patent literature (see, *e.g.*, Rammensee (1995) *Immunogenetics* 41:178-228; Sinigaglia (1994) *Curr. Opin. Immunol.* 6:52-56).

In one embodiment of the invention, the Class II:peptide complexes are water soluble. Water solubility can be engineered in Class II polypeptides by deleting transmembrane domain (typically hydrophobic) amino acid residues. This is most effectively accomplished by recombinantly redesigning the DR allele and expressing the truncated Class II molecule. Deletion of the transmembrane domain can be by outright deletion of residues or by recombinantly redesigning Class II coding sequence to substitute hydrophobic residues with hydrophilic residues. This also facilitates recovery of the water-soluble Class II polypeptide after recombinant expression. For example, the transmembrane-inactivated Class II can be secreted directly into the culture medium of recombinant expression hosts. Further deletion of cytoplasmic or extracellular residues can be effective in eliminating potentially immunogenic epitopes. In one embodiment, a nucleic acid is designed to recombinantly express a minimal peptide binding site/TCR binding polypeptide, *i.e.*, only the amount of polypeptide needed to bind the MBP peptide of the invention with sufficient affinity to be recognized by the appropriate autoreactive TCR and to be used in the methods of the invention. The peptide-binding polypeptide, whether in the form of a minimal peptide binding site molecule, an isolated or recombinant full length, alterred or truncated Class II molecule, or the like, can be in dimeric or moneric form.

Additional Class II polymorphic DR alleles can be identified and characterized using various methods, including: i) computer searches of DNA databases for DNAs containing sequences conserved with the Class II DR molecules described above, ii) hybridization with a probe from a known DR gene sequence to mRNA, cDNA or DNA sequence or libraries, and, iii) by PCR or other signal or target amplification technologies using primers complementary to regions highly conserved among different DR genes.

Nucleic acid amplification methods can also used to identify, isolate and generate Class II polymorphic alleles. Suitable amplification methods include, but are not limited to: polymerase chain reaction, PCR (PCR PROTOCOLS, A GUIDE TO METHODS AND APPLICATIONS, *ed.* Innis, Academic Press, N.Y. (1990) and PCR STRATEGIES (1995), *ed.*

Innis, Academic Press, Inc., N.Y. (Innis)); ligase chain reaction (LCR) (Wu (1989) *Genomics* 4:560; Landegren (1988) *Science* 241:1077; Barringer (1990) *Gene* 89:117); transcription amplification (Kwoh (1989) *Proc. Natl. Acad. Sci. USA* 86:1173); and, self-sustained sequence replication (Guatelli (1990) *Proc. Natl. Acad. Sci. USA,* 87:1874); Q Beta replicase amplification (Smith (1997) *J. Clin. Microbiol.* 35:1477-1491, automated Q-beta replicase amplification assay; Burg (1996) *Mol. Cell. Probes* 10:257-271) and other RNA polymerase mediated techniques (*e.g.*, NASBA, Cangene, Mississauga, Ontario); see also Berger (1987) *Methods Enzymol.* 152:307-316, Sambrook, Tijssen, and Ausubel, as well as Mullis (1987) U.S. Patent Nos. 4,683,195 and 4,683,202.

Sequencing of Nucleic Acid

Sequencing of isolated MBP- and Class II- encoding nucleic acid is used, *e.g.*, to identify and characterize allelic DR species encoding polypeptides capable of binding MBP peptides; to confirm sequences of synthetically manufactured or cloned nucleic acid; confirm mutations; and the like. MBP- and Class II-encoding sequences can be sequenced as inserts in vectors, as inserts released and isolated from the vectors or in any of a variety of other forms (*i.e.*, as amplification products). MBP- and Class II-encoding inserts can be released from the vectors by restriction enzymes or amplified by PCR or transcribed by a polymerase. For sequencing of the inserts to identify full length coding sequences, primers based on the N- or C- terminus, or based on insertion points in the original phage or other vector, can be used. A variety of nucleic acid sequencing techniques are well known and described in the scientific and patent literature, see, *e.g.* Rosenthal (1987) *supra*; Arlinghaus (1997) *Anal. Chem.* 69:3747-3753; Dubiley (1997) *Nucleic Acids Res.* 25:2259-2265; for use of biosensor chips for identification and sequencing of nucleic acids.

Expression of Recombinant MBP Peptides and Class II Polypeptides

The invention provides methods and reagents for recombinant expression of the novel MBP peptides and Class II molecules used in the complexes of the invention. The nucleic acids of the invention may be introduced into a genome or into the cytoplasm or a nucleus of a cell and expressed by a variety of conventional techniques, well described in the scientific and patent literature. See, *e.g.*, Roberts (1987) *Nature* 328:731; Berger (1987) *supra*; Schneider (1995) *Protein Expr. Purif.* 6435:10; Sambrook, Tijssen or Ausubel. Product information from manufacturers of biological reagents and experimental equipment

also provide information regarding known biological methods. The promoters and vectors used in this invention can be isolated from natural sources, obtained from such sources as ATCC or GenBank libraries, or prepared by synthetic or recombinant methods, as described herein. A few selected illustrative general and specific teaching examples relevant to such technology are described below.

Vectors and Transcriptional Control Elements

The invention, providing methods and reagents for making the novel nucleic acids described herein, further provides methods and reagents for expressing these nucleic acids using novel expression cassettes, vectors, transgenic plants and animals, using constitutive and inducible transcriptional and translational *cis*- (*e.g.*, promoters and enhancers) and *trans*-acting control elements. The expression of natural, recombinant or synthetic MBP peptide- or polypeptide-encoding nucleic acids can be achieved by operably linking the coding region a promoter (that can be tissue-specific, constitutive or inducible), incorporating the construct into an expression cassette (such as an expression vector), and introducing the resultant construct into an *in vitro* reaction system or a suitable host cell or organism. Synthetic procedures may also be used to generate any nucleic acid of the invention. Transcriptional and translational control elements include transcription and translation initiation sequences, promoters and enhancers, transcription and translation terminators, polyadenylation sequences, and other sequences useful for transcribing DNA into RNA. In construction of recombinant expression cassettes, vectors, transgenics, of the invention, a promoter fragment can be employed to direct expression of the desired nucleic acid in all tissues of a plant or animal. Promoters that drive expression continuously under physiological conditions are referred to as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. The expression systems optionally at least one independent terminator sequence, sequences permitting replication of the cassette *in vivo, e.g.*, plants, eukaryotes, or prokaryotes, or a combination thereof, (*e.g.*, shuttle vectors) and selection markers for the selected expression system, *e.g.*, plant, prokaryotic or eukaryotic systems. To ensure proper polypeptide expression under varying conditions, a polyadenylation region at the 3'-end of the coding region can be included.

The nucleic acids of the invention can be expressed in expression cassettes, vectors or viruses which are transiently expressed in cells using, *e.g.*, episomal expression

systems. Expression vectors capable of expressing proteins are well known in the art. Selection markers can be incorporated into expression cassettes and vectors to confer a selectable phenotype on transformed cells and sequences coding for episomal maintenance and replication such that integration into the host genome is not required. For example, the marker may encode antibiotic resistance, particularly resistance to chloramphenicol, kanamycin, G418, bleomycin, hygromycin, or herbicide resistance, such as resistance to chlorosulfuron or Basta, to permit selection of those cells transformed with the desired DNA sequences, see for example, Blondelet-Rouault (1997) *Gene* 190:315-317; Aubrecht (1997) *J. Pharmacol. Exp. Ther.* 281:992-997 . Because selectable marker genes conferring resistance to substrates like neomycin or hygromycin can only be utilized in tissue culture, chemoresistance genes are also used as selectable markers *in vitro* and *in vivo*.

Production of Transformants and Transgenic Plants and Animals

The invention provides for a variety of *in vivo* systems expressing the MBP peptides, Class II polypeptides, and peptide:Class II complexes of the invention, including transformed cells and transgenic plants and animals. *In vivo* expression systems that can be used include bacterial, yeast, insect (baculovirus), plant, and mammalian cell systems. The system used will depend on a variety of factors, including activities and amounts desired.

There are several well-known methods of introducing nucleic acids into animal, plant, bacterial and other cells, a process often called "transforming," any of which may be used in the methods of the present invention (see, *e.g.*, Sambrook, Ausubel or Tijssen). *In vivo* expression systems and techniques for transforming a wide variety of animal and plant cells are well known and described in the technical and scientific literature. See, *e.g.*, Weising, *Ann. Rev. Genet.* 22:421-477 (1988) for plant cells and Sambrook or Tijssen for animal and bacterial cells.

II. MBP Peptides and MHC Class II DR Polypeptides

This invention provides novel myelin basic protein (MBP) peptides, Class II polypeptides comprising at least an amount of polypeptide needed to bind an MBP peptide of the invention with sufficient affinity to be recognized by the appropriate autoreactive TCR, and Class II:MBP peptide complexes. The invention provides isolated (from natural sources), synthetic, and recombinantly generated forms of MBP peptides and Class II polypeptides.

These peptides and proteins can be recombinantly expressed *in vitro* or *in vivo*. The peptides, polypeptides and complexes of the invention can be made and isolated using any method known in the art, and the invention provides a few exemplary means for generating such proteins. In addition, means to make Class II:peptide complexes are taught in, *e.g.*, U.S. Patent Nos. (USPN) 5,194,425, issued March 16, 1993; 5,130,297, issued July 14, 1992; 5,284,935, issued February 8, 1994; 5,260,422, issued November 9, 1993; and 5,468,481, issued November 21, 1995.

MBP peptides and Class II:peptide complexes of the invention can be synthesized, whole or in part, using chemical methods well known in the art (see *e.g.*, Caruthers (1980) *Nucleic Acids Res. Symp. Ser.* 215-223; Horn (1980) *Nucleic Acids Res. Symp. Ser.* 225-232; Banga, A.K., *Therapeutic Peptides and Proteins, Formulation, Processing and Delivery Systems* (1995) Technomic Publishing Co., Lancaster, PA ("Banga")). For example, peptide synthesis can be performed using various solid-phase techniques (see *e.g.*, Roberge (1995) *Science* 269:202; Merrifield (1997) *Methods Enzymol.* 289:3-13) and automated synthesis may be achieved, *e.g.*, using the ABI 431A Peptide Synthesizer (Perkin Elmer) in accordance with the instructions provided by the manufacturer.

The newly synthesized peptide can be isolated and substantially purified by preparative high performance liquid chromatography (HPLC), see *e.g.*, Creighton, *Proteins, Structures and Molecular Principles*, WH Freeman and Co, New York NY, 1983. The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (*e.g.*, the Edman degradation procedure; Creighton, *supra*). Laser desorption mass spectrometry (MALDI-MS) can also be used to evaluate the progress of peptide synthesis at all the necessary levels, including automated assembly, cleavage and deprotection chemistries, RP-HPLC analyses and purifications, and structural validation of the final product (Moore (1997) *Methods Enzymol.* 289:520-542). Electrospray ionization mass spectrometry is an easy, rapid method for the verification of proper peptide synthesis and for the identification of most synthetic by-products (Burdick (1997) *Methods Enzymol.* 289:499-519).

Additionally the amino acid sequences of the peptides and polypeptides of the invention, or any part thereof, can be alterred during direct synthesis and/or combined using chemical methods with sequences from other proteins, or any part thereof, to produce a variant polypeptide. Modified peptides and proteins of the invention can be produced by, in addition

to manipulation of nucleic acid coding sequence, *e.g.*, with site-directed mutagenesis, chemical modification of polypeptide to introduce unnatural amino acid side chains (see *e.g.*, Paetzel (1997) *J. Biol. Chem.* 272:9994-10003, for general methodology). As another example, for site-specific incorporation of biotin-containing amino acid biocytin, see Gallivan (1997) *Chem. Biol.* 4:739-749; for site-specific incorporation of unnatural amino acids into proteins *in vivo*, see *e.g.*, Liu (1997) *Proc. Natl. Acad. Sci. USA* 94:10092-10097; see also Koh (1997) *Biochemistry* 36:11314-11322.

Class II polypeptides suitable for use in the present invention can also be isolated from a natural sources, such as a cell line expressing the appropriate DR allele or a patient of the appropriate genotype, using a variety of techniques well known in the art. For example, the cells can be solubilized by treatment with papain, by treatment with 3M KCl, or by treatment with detergent. Detergent extraction of Class II protein from lymphocytes followed by affinity purification can also be used. Detergent can then be removed by dialysis or selective binding beads, *e.g.*, Bio Beads. The molecules can be obtained by isolation from any cell expressing the Class II molecule of interest, such as, *e.g.*, B or T lymphocytes from an individual with the appropriate genotype, as one suffering from demyelinating autoimmune disease. Suitable MHC molecules can be isolated from B or T cells which have been immortalized by transformation, *e.g.*, as by B cell transformation with a replication deficient Epstein-Barr virus, utilizing techniques known in the art.

Isolation of individual subunits from the isolated Class II molecule is easily achieved using standard techniques known to those skilled in the art. For example, alpha and beta subunits from Class II molecules can be separated using SDS/PAGE and electroelution. (see *e.g.*, Rothenhausler (1990) *Proc. Natl. Acad. Sci. USA* 87:352-354; Gorga (1987) *J. Biol. Chem.* 262:16087-16094; Dommair (1989) *Cold Spring Harbor Symp. Quant. Biol.* 54:409-416). One of skill will recognize that a number of other standard methods of separating molecules can be used, such as, *e.g.*, ion exchange chromatography, size exclusion chromatography, gel permeation chromatography, HPLC, RP-HPLC, or affinity chromatography. See, *e.g.*, Banga.

In one embodiment, an additional "effector" composition is linked to the complexes of the invention to inhibit or abrogate the autoimmune reaction. The "effector" portion of the molecule can be, *e.g.*, a toxin, a chemotherapeutic agent, an antibody to a

cytotoxic T lymphocyte (CTL) surface molecule, a lipase, or a toxic radioisotope emitting, *e.g.*, gamma radiation from radioisotopes such as yttrium-90, phosphorus-32, lead-212, iodine-131, or palladium-109. A number of protein toxins are well known in the art including, *e.g.*, ricin, diphtheria, gelonin, *Pseudomonas* toxin, and abrin. Chemotherapeutic agents include, *e.g.*, doxorubicin, daunorubicin, methotrexate, cytotoxin, and anti-sense RNA. Antibiotics can also be used. In some cases the toxin or other effector component is entrapped in a delivery system such as a liposome or dextran carrier; in these cases, either the active component or the carrier may be bound to the Class II:peptide complex.

In preparing pharmaceutical compositions of the present invention, it is frequently desirable to modify the peptides or complexes to alter their pharmacokinetics and biodistribution (discused further below). For instance, methods suitable for increasing serum half-life of the complexes include treatment to remove carbohydrates which are involved in the elimination of the complexes from the bloodstream. Preferably, substantially all of the carbohydrate moieties are removed by the treatment. Substantially all of the carbohydrate moieties are removed if at least about 75%, preferably about 90%, and most preferably about 99% of the carbohydrate moieties are removed. Conjugation to soluble macromolecules, such as proteins, polysaccharides, or synthetic polymers, such as polyethylene glycol, is also effective. Other methods include protection of the complexes in vesicles composed of substances such as proteins, lipids (for example, liposomes, discussed below), carbohydrates, or synthetic polymers.

Formation of the Complex

The Class II polypeptide:MBP peptide complex can be formed by any standard means known in the art. For example, the peptides of the invention can be associated noncovalently with antigen binding sites by, *e.g.*, simply mixing the two components. They can also be covalently bound to the antigen binding pocket using standard procedures by, *e.g.*, photoaffinity labelling (see, *e.g.*, Hall (1985) *Biochemistry* 24:5702-5711; Leuscher (1990) *J. Biol. Chem.* 265:11177-11184; Wraith (1989) Cell 59:247-255). Other modes of linkage include, *e.g.*, attachment via carbohydrate/lectin groups on the glycoproteins (Husain (1995) *Biochem. Mol. Biol. Int.* 36:669-677), as the carbohydrate moieties of Class II alpha and/or beta chains. Dehydration reactions using carbodiimides can be used. Heterobifunctional linkers such as N-hydroxysuccinimide ester (NHS), N-succinimidyl 3-(2-pyridyldithio)-

propionate (SPDP), substituted 2-iminothiolanes, glutaraldehyde and the like can also be used (see, *e.g.*, Traut (1995) *Biochem. Cell Biol.* 73:949-958; Haselgrubler (1995) *Bioconjug Chem.* 6:242-248; Carroll (1994) *Bioconjug. Chem.* 5:248-256). Alternatively, the Class II: peptide complex can be designed as one contiguous recombinant polypeptide. See also, PCT Publication Nos. WO 96/40944, December 19, 1996; WO 96/40194, December 19, 1996; and, WO 97/04360, November 6, 1997.

Effectors or other fusion protein components (for purification, etc., discussed above) can also be attached to the complex using these methods. The sequence of preparing the complex depends on the components in each instance. For example, in an exemplary protocol, the peptide portion and MHC subunit component are noncovalently associated by contacting the peptide with the MHC subunit component, *e.g.*, by mixing. The effector is then covalently linked. Alternatively, the effector and MHC subunit may be first conjugated and this conjugate complexed with the MBP peptide component. If the effector is itself a protein, the entire complex may be made directly from the appropriate encoding DNA using recombinant methods.

Assessment of the Complex

The peptides and MBP:Class II complexes of the invention can be assayed using a variety of *in vitro* models well known in the art. As discussed above, to activate CD4+ T cells, TCR binding by Class II:peptide is not sufficient. An additional, "co-stimulatory" signal is needed. Interaction of a Class II:peptide complex of the invention with TCR lacks a co-stimulatory signal. Thus, a state of antigen-specific T cell non-responsiveness is induced (Boussiotis (1994) *Curr. Opin. Immunol.* 6:797-807; Park (1997) *Eur. J. Immunol.* 27:1082-1090). This "tolerance" or "anergy" immunosuppression and re-challenge non-responsiveness can be caused by T cell clonal anergy, by a nonresponsiveness induced by immunosuppressive cytokines, or both (Schwartz (1989) *Cell* 57:1073-1081; Quill (1987) *J. Immunol.* 138:3704-3712).

In one exemplary *in vitro* system, the complex is incubated with autoreactive T cells and the cells are rechallenged with MBP. Peripheral blood T cells from patients with a demyelinating disease, such as MS, or myelin- (MBP-) reactive T cell clones can be used. T cells can be isolated before the analysis. The autoreactive T cells can be restimulated with antigen (MBP or myelin) *in vitro* (using syngeneic APCs) before administration of the

putative toleragen. "Resting" T cells (cells not stimulated *in vitro*) can be used. The T cells are treated with varying amounts of a complex of the invention for varying amounts of time. The binding of the complexes to the MBP-reactive T cells results in inhibiting or abrogating the *in vitro* autoimmune reaction.

The degree of immunosuppression or re-challenge non-responsiveness (i.e., tolerance, anergy), can be measured by monitering cell proliferation, cell metabolism, secretion of cytokines or lymphokines, or any form of cell activation. T cell activation can be measured by a variety of means well known in the art. For example, T cell proliferation can be assessed, *e.g.*, as measured by ^{3}H-thymidine uptake or by uptake of 3-(4,5-dimethyl-thiazol-2-7')-2,5 diphenyltetrazolium bromide (MTT) (see, *e.g.*, Liu (1997) *J. Neurochem.* 69:581-593). Alternatively, as T cells synthesize and secrete cytokines upon activation, the immunosuppressive efficacy of a Class II:peptide complex can be assessed by measuring cytokine transcription, translation or secretion. Thus, a variety of cytokines and lymphokines can be quantitated, *e.g.*, interleukins, interferons (INFs) (*e.g.*, gamma INF), tumor necrosis factors (TNFs) (*e.g.*, TNF beta) and the like. Cell death can also be monitered, as it has been observed that prolonged incubation of resting T cells with soluble MHC-Class II peptide complexes results in T cell apoptosis (Arimilli (1996) *Immunol. and Cell Biol.* 74:96-104). Cell death can be measured by a variety of known procedures, *e.g.*, dye exclusion permeability. Apoptosis can be assessed using, *e.g.*, cellular DNA fragmentation, observation (as with transmission electron microscopy), detection and quantitation of apoptosis-associating protein, as bcl-2, and the like (see, *e.g.*, Arimilli (1996) *supra*).

A preferred method of assessing the immunosuppressive efficacy of the Class II:peptide complexes of the invention is through the use of a microphysiometer to measure the rate of production of acidic metabolites in T cells. By using this apparatus, effect of the interaction of the complexes with TCRs can be quickly and easily detected. The earliest measurable event in the activation of a T cell after stimulation by a TCR:Class II:peptide complex binding reaction is an increase in the lymphocyte's metabolism, as reflected by its acidic byproducts. The microphysiometer measures the acidity of the principal catabolic products in mammalian cells, lactate and carbon dioxide. Very small changes in the acidity of the cultural medium bathing a small sample of cells can be readily determined with a light-addressable potentiometric sensor. The rate of acidification is used as a measure of catabolic

rate of the cells being assayed. See, *e.g.*, Parce (1989) *Science* 246:243-247; Owicki (1990) *Proc. Natl. Acad. Sci. USA* 87:4007-4011; Renschler (1995) *Cancer Res.* 55:5642-5647; Beeson (1996) *J. Exp. Med.* 184:777-782.

Addition of MBP peptide or myelin polypeptide to a mixture of autoreactive T cells and syngeneic APCs results in an increase in acid release due to the antigen specific binding of autoreactive TCR and APC-bound Class II:MBP peptide complexes. The immunosuppressive capability of an MBP peptide or Class II:MBP peptide complex of the invention can thus be evaluated by first adding a complex to an autoreactive T cell/APC culture followed by antigen (MBP or myelin) challenge. Lack of or a relative decrease in cell activation indicative of immunosuppression can be measured by a lessening or lack of extracellular acidification.

In one embodiment, a complex of the invention with an effector component is used. The treatment can be two-fold: the individual is first treated with the Class II:MBP peptide complex to down-regulate the immune system; further down-regulation is achieved by treatment with complex and an effector component.

III. Detection and Purification of Polypeptides

The invention also provides methods and reagents for isolating, detecting or quantitating the MBP peptides and Class II polypeptide complexes of the invention by a variety of methods.

Antibodies

In one embodiment, the invention provides antibodies specifically immunoreactive under immunologically reactive conditions to an MBP peptide of the invention. These Abs can be used in the isolation, detection or quantitation of the MBP peptides or complexes of the invention. Methods of producing polyclonal and monoclonal antibodies are known to those of skill in the art and described in the scientific and patent literature, see, *e.g.*, Coligan, CURRENT PROTOCOLS IN IMMUNOLOGY, Wiley/Greene, NY (1991); Stites (eds.) BASIC AND CLINICAL IMMUNOLOGY (7th ed.) Lange Medical Publications, Los Altos, CA ("Stites"); Goding, MONOCLONAL ANTIBODIES: PRINCIPLES AND PRACTICE (2d ed.) Academic Press, New York, NY (1986); Kohler (1975) *Nature* 256:495; Harlow (1988) ANTIBODIES, A LABORATORY MANUAL, Cold Spring Harbor Publications, New York. Such

techniques include selection of antibodies from libraries of recombinant antibodies displayed in phage ("phage display libraries") or similar on cells. *See*, Huse (1989) *Science* 246:1275; Ward (1989) *Nature* 341:544; Hoogenboom (1997) *Trends Biotechnol.* 15:62-70; Katz (1997) *Annu. Rev. Biophys. Biomol. Struct.* 26:27-45. Recombinant antibodies can be expressed by transient or stable expression vectors in mammalian cells, as in Norderhaug (1997) *J. Immunol. Methods* 204:77-87; Boder (1997) "Yeast surface display for screening combinatorial polypeptide libraries," *Nat. Biotechnol.* 15:553-557.

Purification of MBP Peptides and Complexes

The methods and reagents of the invention enable one to purify the MBP peptides and complexes of the invention from a variety of sources, depending on which natural source, synthetic or recombinant expression system selected, such as plant cells, larval homogenates, bacterial cells, yeast, mammalian cells, human cells, tissue culture media, transgenic plants and animals, to substantial purity. General information relating to standard purification procedures, is well known in the patent and scientifice literature, as described *supra*; see also, *e.g.*, Scopes, R. K., Protein Purification: Principles and Practice, 2nd ed., Springer Verlag, (1987), Banga, Ausubel, and Sambrook.

Fusion Proteins

MBP peptides and complex polypeptides can also be expressed as proteins with one or more additional polypeptide domains linked thereto to facilitate cell killing (using, *e.g.*, effector agents, as described above), protein detection, purification, or other applications. Detection and purification facilitating domains include, *e.g.*, metal chelating peptides such as polyhistidine tracts and histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle WA). The inclusion of a cleavable linker sequences such as Factor Xa or enterokinase (Invitrogen, San Diego CA) between the purification domain and plant disease resistant polypeptide may be useful to facilitate purification. For example, one expression vector includes a polypeptide-encoding nucleic acid sequence linked to six histidine residues followed by a thioredoxin and an enterokinase cleavage site (*e.g.*, see Williams (1995) *Biochemistry* 34:1787-1797). The histidine residues facilitate detection and purification while the enterokinase cleavage site provides a means for purifying the desired protein(s) from the

remainder of the fusion protein. Technology pertaining to vectors encoding fusion proteins and application of fusion proteins are well described in the scientific and patent literature, see *e.g.*, Kroll (1993) *DNA Cell. Biol.*, 12:441-53.

IV. Formulation and Administration of MBP Peptides and Complexes: Pharmaceutical Compositions

The MBP peptides and Class II:peptide complexes of the invention are typically combined with a pharmaceutically acceptable carrier (excipient) to form a pharmacological composition. Pharmaceutically acceptable carriers can contain a physiologically acceptable compound that acts to, *e.g.*, stabilize, or increase or decrease the absorption or clearance rates of the pharmaceutical compositions of the invention. Physiologically acceptable compounds can include, *e.g.*, carbohydrates, such as glucose, sucrose, or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins, compositions that reduce the clearance or hydrolysis of the peptides or polypeptide complexes, or excipients or other stabilizers and/or buffers. Detergents can also used to stabilize or to increase or decrease the absorption of the pharmaceutical composition, see *infra* for exemplary detergents, including liposomal carriers. Pharmaceutically acceptable carriers and formulations for peptides and polypeptide are known to the skilled artisan and are described in detail in the scientific and patent literature, see *e.g.*, the latest edition of Remington's Pharmaceutical Science, Mack Publishing Company, Easton, Pennsylvania ("Remington's") and Banga.

Other physiologically acceptable compounds include wetting agents, emulsifying agents, dispersing agents or preservatives which are particularly useful for preventing the growth or action of microorganisms. Various preservatives are well known and include, *e.g.*, phenol and ascorbic acid. One skilled in the art would appreciate that the choice of a pharmaceutically acceptable carrier including a physiologically acceptable compound depends, for example, on the route of administration of the protein or polypeptide of the invention and on its particular physio-chemical characteristics.

Aqueous Solutions for Enteral, Parenteral Or Transmucosal Administration

The compositions for administration will commonly comprise a solution of the peptide or polypeptide of the invention dissolved in a pharmaceutically acceptable carrier,

preferably an aqueous carrier if the composition is water-soluble. Examples of aqueous solutions that can be used in formulations for enteral, parenteral or transmucosal drug delivery include, *e.g.*, water, saline, phosphate buffered saline, Hank's solution, Ringer's solution, dextrose/saline, glucose solutions and the like. The formulations can contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as buffering agents, tonicity adjusting agents, wetting agents, detergents and the like. Additives can also include additional active ingredients such as bactericidal agents, or stabilizers. For example, the solution can contain sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate or triethanolamine oleate. These compositions can be sterilized by conventional, well-known sterilization techniques, or can be sterile filtered. The resulting aqueous solutions can be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The concentration of MBP peptide and/or Class II:peptide complex in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs.

Solid Formulations For Enteral Delivery

Solid formulations can be used for enteral (oral) administration. They can be formulated as, *e.g.*, pills, tablets, powders or capsules. For solid compositions, conventional nontoxic solid carriers can be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10% to 95% of active ingredient (MBP peptide or complex). A non-solid formulation can also be used for enteral administration. The carrier can be selected from various oils including those of petroleum, animal, vegetable or synthetic origin, *e.g.*, peanut oil, soybean oil, mineral oil, sesame oil, and the like. Suitable pharmaceutical excipients include *e.g.*, starch, cellulose, talc, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, magnesium stearate, sodium stearate, glycerol monostearate, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol, and the like.

It is recognized that the MBP peptides and polypeptide complexes of the invention, when administered orally, must be protected from digestion. This is typically accomplished either by complexing the peptide or polypeptide complex with a composition to render it resistant to acidic and enzymatic hydrolysis or by packaging the peptide or complex in an appropriately resistant carrier such as a liposome. Means of protecting compounds from digestion are well known in the art, *see, e.g.,* Fix (1996) *Pharm Res.* 13:1760-1764; Samanen (1996) *J. Pharm. Pharmacol.* 48:119-135; U.S. Patent 5,391,377, describing lipid compositions for oral delivery of therapeutic agents (liposomal delivery is discussed in further detail, *infra*).

Topical Formulations For Transdermal/ Transmucosal Delivery

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated can be used in the formulation. Such penetrants are generally known in the art, and include, *e.g.*, for transmucosal administration, bile salts and fusidic acid derivatives. In addition, detergents can be used to facilitate permeation. Transmucosal administration can be through nasal sprays or using suppositories. See, *e.g.*, Banga, Chapt. 10; Sayani (1996) "Systemic delivery of peptides and proteins across absorptive mucosae" *Crit. Rev. Ther. Drug Carrier Syst.* 13:85-184. For topical, transdermal administration, the agents are formulated into ointments, creams, salves, powders and gels. Transdermal delivery systems can also include, *e.g.*, patches. See, *e.g.*, Banga, Chapt. 9.

The peptides and polypeptide complexes can also be administered in sustained delivery or sustained release mechanisms, which can deliver the formulation internally. For example, biodegradeable microspheres or capsules or other biodegradeable polymer configurations capable of sustained delivery of a composition (*e.g.*, an MBP peptide:Class II polypeptide complex) can be included in the formulations of the invention (see, *e.g.*, Putney (1998) *Nat. Biotechnol.* 16:153-157).

Formulations for Inhalation Delivery

For inhalation, the peptide or polypeptide can be delivered using any system known in the art, including dry powder aerosols, liquids delivery systems, air jet nebulizers, propellant systems, and the like. See, *e.g.*, Patton (1998) *Biotechniques* 16:141-143; product and inhallation delivery systems for polypeptide macromolecules by, *e.g.*, Dura

Pharmaceuticals (San Diego, CA) , Aradigm (Hayward, CA), Aerogen (Santa Clara, CA), Inhale Therapeutic Systems (San Carlos, CA), and the like.

For example, the pharmaceutical formulation can be administered in the form of an aerosol or mist. For aerosol administration, the formulation can be supplied in finely divided form along with a surfactant and propellant. The surfactant preferably is soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride such as, for example, ethylene glycol, glycerol, erythritol, arabitol, mannitol, sorbitol, the hexitol anhydrides derived from sorbitol, and the polyoxyethylene and polyoxypropylene derivatives of these esters. Mixed esters, such as mixed or natural glycerides can be employed. The surfactant can constitute 0.1% to 20% by weight of the composition, preferably 0.25% to 5%. The balance of the formulation is ordinarily propellant. Liquefied propellants are typically gases at ambient conditions, and are condensed under pressure. Among suitable liquefied propellants are the lower alkanes containing up to 5 carbons, such as butane and propane; and preferably fluorinated or fluorochlorinated alkanes. Mixtures of the above can also be employed. In producing the aerosol, a container equipped with a suitable valve is filled with the appropriate propellant, containing the finely divided compounds and surfactant. The ingredients are thus maintained at an elevated pressure until released by action of the valve. See, *e.g.*, Edwards (1997) "Large porous particles for pulmonary drug delivery" *Science* 276:1868-1871.

In another embodiment, the device for delivering the formulation to respiratory tissue is an inhaler in which the formulation vaporizes. Other liquid delivery systems include, *e.g.*, air jet nebulizers.

Other Formulations

In preparing pharmaceuticals of the present invention, a variety of formulation modifications can be used and manipulated to alter pharmacokinetics and biodistribution. A number of methods for altering pharmacokinetics and biodistribution are known to one of ordinary skill in the art. Examples of such methods include protection of the complexes in vesicles composed of substances such as proteins, lipids (for example, liposomes, see below),

carbohydrates, or synthetic polymers (discussed above). For a general discussion of pharmacokinetics, see, *e.g.,* Remington's, Chapters 37-39, or Banga, Chapt. 6.

Routes of Delivery

The peptide and polypeptide complexes used in the methods of the invention can be delivered alone or as pharmaceutical compositions by any means known in the art, *e.g.*, systemically, regionally, or locally; by intraarterial, intrathecal (IT), intravenous (IV), parenteral, intra-pleural cavity, topical, oral, or local administration, as subcutaneous, intra-tracheal (*e.g.,* by aerosol) or transmucosal (*e.g.*, buccal, bladder, vaginal, uterine, rectal, nasal mucosa). Actual methods for preparing administrable compositions will be known or apparent to those skilled in the art and are described in detail in the scientific and patent literature, see *e.g.*, Remington's or Banga. Particularly preferred modes of administration include intra-arterial or intrathecal (IT) injections, especially when it is desired to have a "regional effect," e.g., to focus on a specific organ, *e.g.*, brain and CNS (see *e.g.*, Gurun (1997) *Anesth Analg.* 85:317-323). For example, intra-carotid artery injection if preferred where it is desired to deliver a peptide or polypeptide complex of the invention directly to the brain. Parenteral administration is a preferred route of delivery if a high systemic dosage is needed. Enteral administration is a preferred method if administration of peptide to induce oral tolerance is the therapeutic objective, see, *e.g.*, Kennedy (1997) *J. Immunol.* 159:1036-1044; Kent (1997) *Ann. NY Acad. Sci.* 815:412-422. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in detail, in *e.g.*, Remington's, Banga Chapt 7. See also, Bai (1997) *J. Neuroimmunol.* 80:65-75; Warren (1997) *J. Neurol. Sci.* 152:31-38; Tonegawa (1997) *J. Exp. Med.* 186:507-515.

Treatment Regimens: Pharmacokinetics

The pharmaceutical compositions can be administered in a variety of unit dosage forms depending upon the method of administration. Dosages for typical peptide and polypeptide pharmaceutical compositions are well known to those of skill in the art. Such dosages are typically advisorial in nature and are adjusted depending on the particular therapeutic context, patient tolerance, *etc.* The amount of MBP peptide or Class II:peptide complex adequate to accomplish this is defined as a "therapeutically effective dose." The dosage schedule and amounts effective for this use, *i.e.*, the "dosing regimen," will depend upon a variety of factors, including the stage of the disease or condition, the severity of the

disease or condition, the general state of the patient's health, the patient's physical status, age, pharmaceutical formulation and concentration of active agent, and the like. In calculating the dosage regimen for a patient, the mode of administration also is taken into consideration. The dosage regimen must also take into consideration the pharmacokinetics, *i.e.*, the pharmaceutical composition's rate of absorption, bioavailability, metabolism, clearance, and the like. See, *e.g.*, the latest Remington's; Egleton (1997) "Bioavailability and transport of peptides and peptide drugs into the brain" *Peptides* 18:1431-1439; Langer (1990) *Science* 249:1527-1533.

In therapeutic applications, compositions are administered to a patient suffering from a demyelinating disease in an amount sufficient to cure or at least partially arrest the disease and/or its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the disease, general state of the patient's health, frequency and routes of administration, clinician's judgement, and the like. For example, in one embodiement, a soluble Class II:peptide complex pharmaceutical composition dosage for intravenous (IV) administration would be about 0.01 mg/hr to about 1.0 mg/hr administered over several hours (typically 1, 3, or 6 hours), which can be repeated for weeks with intermittant cycles. Considerably higher dosages (*e.g.*, ranging up to about 10 mg/ml) can be used, particularly when the drug is administered to a secluded site and not into the blood stream, such as into a body cavity or into a lumen of an organ, *e.g.*, the cerebrospinal fluid (CSF).

Dosages can be be determined empirically, by assessing the abatement or amelioration of symptions, or by objective criteria, such analysis of blood or histopathology specimens. For example, in MS, the disease is characterized by various complaints and findings of CNS dysfunction, with remissions and persistently recurring exacerbations. Onset usually is insidious. The most frequent presenting symptoms paresthesias in one or more extremities, in the trunk, or on one side of the face; weakness or clumsiness of a leg or a hand; or visual disturbances (*e.g.*, partial blindness and pain in one eye, diplopia, dimness of vision, scotomas). Other common early symptoms are a fleeting ocular palsy, transient weakness of one or more extremities, slight stiffness or unusual fatigability of a limb, minor gait disturbances, difficulties with bladder control, vertigo, or mild emotional disturbances; all are evidence of scattered CNS involvement and often occurring months or years before the disease

is recognized. Successful treatment can also be monitered by histopathology. In MS, plaques or islands of demyelination with destruction of oligodendroglia and perivascular inflammation are disseminated through the CNS, primarily in the white matter, with a predilection for the lateral and posterior columns (especially in the cervical and dorsal regions), the optic nerves, and periventricular areas. Tracts in the midbrain, pons, and cerebellum also are affected, and gray matter in both cerebrum and cord may be affected. Cell bodies and axons usually are preserved, especially in early lesions. Later, axons may be destroyed, especially in the long tracts, and a fibrous gliosis gives the tracts their "sclerotic" appearance. Both early and late lesions may be found simultaneously. Thus, the compositions of the invention are administered to arrest the progress of the disease and to reduce the onset, frequency or severity of these or other symptoms.

In one exemplary embodiment, the dosage is between about 0.5 mg/kg and about 25 mg/kg, with a preferred embodiment of about 3 mg/kg to about 15 mg/kg. In another exemplary embodiment, a unit dosage is between about 0.01 to 1000 mg per dose, with a preferred embodiment of about 10 to about 100 mg per dose. See also USPN 5,468,481, issued November 21, 1995. In another embodiment, peptide is adminstered enterally to induce oral tolerance in a dosage ranging from 10 to 2500 ug per day, with a preferred embodiment of about 20 to 50 ug per day. See, *e.g.*, Barnett (1998) *Arthritis Rheum.* 41:290-297, where oral administration of cartilage-derived type II collagen (to ameliorate arthritis) at a dosage ranges of 20, 100, 500, or 2,500 ug/day showed best results using 20 ug per day.

The pharmaceutical compositions containing the peptide and complexes of the invention can be administered alone or in conjunction with other therapeutic treatments. Single or multiple administrations of the compositions may be administered depending on the dosage and frequency as required and tolerated by the patient.

Liposomal Formulations

The invention provides pharmaceuticals in which the transmembrane region of the Class II subunit of the complex is included. In one embodiment, the pharmaceutical formulations comprising this Class II:MBP peptide complex are incorporated in lipid monolayers or bilayers. The invention also provides formulations in which water soluble peptides or complexes have been attached to the surface of the monolayer or bilayer. For example, peptides can be attached to hydrazide- PEG- (distearoylphosphatidyl) ethanolamine-

containing liposomes (see, *e.g.*, Zalipsky (1995) *Bioconjug. Chem.* 6:705-708). Liposomes or any form of lipid membrane, such as planar lipid membranes or the cell membrane of an intact cell, *e.g.*, a red blood cell, can be used. Liposomal formulations can be by any means, including administration intravenously, transdermally (see, *e.g.*, Vutla (1996) *J. Pharm. Sci.* 85:5-8), transmucosally, or orally. The invention also provides pharmaceutical preparations in which the peptides and/or complexes of the invention are incorporated within micelles and/or liposomes (see, *e.g.*, Suntres (1994) *J. Pharm. Pharmacol.* 46:23-28; Woodle (1992) *Pharm. Res.* 9:260-265).

Liposomes and liposomal formulations can be prepared according to standard methods and are also well known in the art, see, *e.g.*, Remington's; Akimaru (1995) *Cytokines Mol. Ther.* 1:197-210; Alving (1995) *Immunol. Rev.* 145:5-31; Szoka (1980) *Ann. Rev. Biophys. Bioeng.* 9:467, U.S. Pat. Nos. 4, 235,871, 4,501,728 and 4,837,028. In one embodiment, liposomes of the present invention typically contain the Class II:peptide complexes positioned on the surface of the liposome in such a manner that the complexes are available for interaction with the TCR. The transmembrane region is usually first incorporated into the membrane at the time of forming the membrane. The liposomes can also be used to target desired drugs (*e.g.* toxins or chemotherapeutic agents) to particular autoreactive T cells.

Liposome charge is an important determinant in liposome clearance from the blood, with negatively charged liposomes being taken up more rapidly by the reticuloendothelial system (Juliano (1975) *Biochem. Biophys. Res. Commun.* 63:651) and thus having shorter half-lives in the bloodstream. Incorporating phosphatidylethanolamine derivatives enhance the circulation time by preventing liposomal aggregation. For example, incorporation of N-(omega-carboxy)acylamido-phosphatidylethanolamines into large unilamellar vesicles of L-alpha-distearoylphosphatidylcholine dramatically increases the *in vivo* liposomal circulation lifetime (see, *e.g.*, Ahl (1997) *Biochim. Biophys. Acta* 1329:370-382). Liposomes with prolonged circulation half-lives are typically desirable for therapeutic and diagnostic uses. For instance, liposomes which can be maintained from 8, 12, or up to 24 hours in the bloodstream are particularly preferred embodiments of the invention.

Typically, the liposomes are prepared with about 5 to 15 mole percent negatively charged phospholipids, such as phosphatidylglycerol, phosphatidylserine or phosphatidyl-inositol. Added negatively charged phospholipids, such as phosphatidylglycerol,

also serve to prevent spontaneous liposome aggregating, and thus minimize the risk of undersized liposomal aggregate formation. Membrane-rigidifying agents, such as sphingomyelin or a saturated neutral phospholipid, at a concentration of at least about 50 mole percent, and 5 to 15 mole percent of monosialylganglioside, may provide increased circulation of the liposome preparation in the bloodstream, as generally described in USPN 4, 837,028.

Additionally, the liposome suspension can include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as alpha-tocopherol and water-soluble iron-specific chelators, such as ferrioxianine, are preferred.

The formulations of the invention can include multilamellar vesicles of heterogeneous sizes. In this method, the vesicle-forming lipids are dissolved in a suitable organic solvent or solvent system and dried under vacuum or an inert gas to form a thin lipid film. If desired, the film may be redissolved in a suitable solvent, such as tertiary butanol, and then lyophilized to form a more homogeneous lipid mixture which is in a more easily hydrated powderlike form. This film is covered with an aqueous solution of the peptide or polypeptide complex and allowed to hydrate, typically over a 15 to 60 minute period with agitation. The size distribution of the resulting multilamellar vesicles can be shifted toward smaller sizes by hydrating the lipids under more vigorous agitation conditions or by adding solubilizing detergents such as deoxycholate. The hydration medium contains the peptide or complex at a concentration which is desired in the interior volume of the liposomes in the final liposome suspension. Typically the drug solution contains between 10 to 100 mg/ml of the peptides or complexes of the invention in a buffered saline solution.

Following liposome preparation, the liposomes can be sized to achieve a desired size range and relatively narrow distribution of liposome sizes. One preferred size range is about 0.2 to 0.4 microns, which allows the liposome suspension to be sterilized by filtration through a conventional filter, typically a 0.22 micron filter. The filter sterilization method can be carried out on a high through-put basis if the liposomes have been sized down to about 0.2 to 0.4 microns. Several techniques are available for sizing liposome to a desired size (see, *e.g.*, U.S. Pat. No. 4,737,323). Sonicating a liposome suspension either by bath or probe sonication produces a progressive size reduction down to small unilamellar vesicles less than about 0.05 microns in size. Homogenization is another method which relies on shearing

energy to fragment large liposomes into smaller ones. In a typical homogenization procedure, multilamellar vesicles are recirculated through a standard emulsion homogenizer until selected liposome sizes, typically between about 0.1 and 0.5 microns, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size discrimination. Extrusion of liposome through a small-pore polycarbonate membrane or an asymmetric ceramic membrane is also an effective method for reducing liposome sizes to a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired liposome size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size.

Even under the most efficient encapsulation methods, the initial sized liposome suspension may contain up to 50% or more complex in a free (nonencapsulated) form. Several methods are available for removing non-entrapped compound from a liposome suspension, if desired for a particular formulation. In one method, the liposomes in the suspension are pelleted by high-speed centrifugation leaving free compound and very small liposomes in the supernatant. Another method involves concentrating the suspension by ultrafiltration, then resuspending the concentrated liposomes in a replacement medium. Alternatively, gel filtration can be used to separate large liposome particles from solute molecules. Following this treatment, the liposome suspension can be brought to a desired concentration for use in, *e.g.*, an intravenous, I.P, transdermal, or transmucosal administration. This involve resuspending the liposomes in a suitable volume of appropritate medium, where the liposomes have been concentrated, for example by centrifugation or ultrafiltration, or concentrating the suspension, where the drug removal step has increased total suspension volume. The suspension is then sterilized by filtration as described above. These liposomes comprising the peptides or Class II:peptide complex can be administered parenterally or locally in a dose which varies according to, *e.g.*, the manner of administration, the drug being delivered, the particular disease being treated, etc.

Micelles are commonly used in the art to increase solubility of molecules having nonpolar regions. One of skill will thus recognize that micelles are useful in compositions of the present invention. Micelles comprising the complexes of the invention are prepared according to methods well known in the art (see, *e.g.*, Remington's, Chap. 20).

Micelles comprising the peptides and/or complexes of the present invention are typically prepared using standard surfactants or detergents. Micelles are formed by surfactants (molecules that contain a hydrophobic portion and one or more ionic or otherwise strongly hydrophilic groups) in aqueous solution. As the concentration of a solid surfactant increases, its monolayers adsorbed at the air/water or glass/water interfaces become so tightly packed that further occupancy requires excessive compression of the surfactant molecules already in the two monolayers. Further increments in the amount of dissolved surfactant beyond that concentration cause amounts equivalent to the new molecules to aggregate into micelles. Suitable surfactants include sodium laureate, sodium oleate, sodium lauryl sulfate, octaoxyethylene glycol monododecyl ether, octoxynol 9 and PLURONIC F-127® (Wyandotte Chemicals Corp.). Preferred surfactants are nonionic polyoxyethylene and polyoxypropylene detergents compatible with IV injection such as PLURONIC F-127®, n-octyl-alpha-D-glucopyranoside, and the like. Phospholipids, such as those described for use in the production of liposomes, can also be used for micelle formation. Since, in some embodiments of the invention, the Class II subunit of the complexes comprise a lipophilic transmembrane region and a relatively hydrophilic extracellular domain, mixed micelles can be formed in the presence of common surfactants or phospholipids and the subunits. The mixed micelles of the present invention can comprise any combination of the subunits, phospholipids and/or surfactants. Thus, the micelles can comprise subunits and detergent, subunits in combination with both phospholipids and detergent, or subunits and phospholipid.

Kits can also be supplied for therapeutic or diagnostic uses. In one embodiment the pharmaceutical formulation of the invention is in a lyophilized form, which can be placed in a container. The complexes, which can also be conjugated to a label or toxin, or unconjugated, are included in the kits with buffers, such as Tris, phosphate, carbonate, etc., stabilizers, biocides, inert proteins, *e.g.*, serum albumin, or the like, and a set of instructions for use. Generally, these materials will be present in less than about 5% wt. based on the amount of complex and usually present in total amount of at least about 0.001% wt. based again on the protein concentration. Frequently, it will be desirable to include an inert extender or excipient to dilute the active ingredients, where the excipient may be present in from about 1% to 99% wt. of the total composition. Where an antibody capable of binding to the complex

is employed in an assay, this will usually be present in a separate vial. The antibody is typically conjugated to a label and formulated according to techniques well known in the art.

DEFINITIONS

To facilitate understanding the invention, a number of terms are defined below.

The term "antibody" refers to a polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments or synthetic or recombinant analogues thereof which specifically bind and recognize analytes and antigens, such as a genus or subgenus of polypeptides of the invention, as described *supra*.

The term "conservative substitution" refers to a change in the amino acid composition of a peptide or protein, such as polypeptide comprising the MBP peptide sequences of the invention, that does not substantially alter the peptide's or protein's activity. This includes conservatively modified variations of a particular amino acid sequence, *i.e.*, amino acid substitutions of those amino acids that are not critical for protein activity or substitution of amino acids with other amino acids having similar properties (*e.g.*, acidic, basic, positively or negatively charged, polar or non-polar, *etc.*) such that the substitutions of even critical amino acids does not substantially alter activity. A polypeptide sequence of the invention implicitly encompasses conservatively substituted variants thereof. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following six groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (a), Serine (S), Threonine (T); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W) (see also, Creighton (1984) *Proteins,* W.H. Freeman and Company). One of skill in the art will appreciate that the above-identified substitutions are not the only possible conservative substitutions. For example, for some purposes, one may regard all charged amino acids as conservative substitutions for each other whether they are positive or negative. In addition, individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids in an encoded sequence can also be considered "conservatively modified variations." The term "conservative substitution" also refers to a change in a nucleic acid sequence such that the substitution does not substantially alter the

contemplated activity of the nucleic acid, for example, as not changing the activity of the peptide or protein encoded by the nucleic acid. A nucleic acid sequence of the invention implicitly encompasses conservatively modified variants thereof (*e.g.* degenerate codon substitutions) and complementary sequences and as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer (1991) *Nucleic Acid Res.* 19:5081; Ohtsuka (1985) *J. Biol. Chem.* 260:2605-2608; Rossolini (1994) *Mol. Cell. Probes* 8:91-98).

A "fusion protein" refers to a composition comprising at least one polypeptide or peptide domain which is associated with a second domain. The second domain can be a polypeptide, peptide, polysaccharide, or the like. The "fusion" can be an association generated by a chemical linking or by a charge (electrostatic attraction, *i.e.*, salt bridges, H-bonding, etc.) interaction. If the polypeptides are recombinant, the "fusion protein" can be translated from a common message. Alternatively, the compositions of the domains can be linked by any chemical or electrostatic means.

A "heterologous sequence" refers to any amino acid or nucleic acid sequence that is not a myelin basic protein sequence.

As used herein, "isolated," when referring to a molecule or composition, such as, for example, an MBP peptide, Class II:peptide complex, or nucleic acid, means that the molecule or composition is separated from at least one other compound, such as a protein, other nucleic acids (*e.g.*, RNAs), or other contaminants with which it is associated *in vivo* or in its naturally occurring state. Thus, a polypeptide or nucleic acid is considered isolated when it has been isolated from any other component with which it is naturally associated, *e.g.*, cell membrane, as in a cell extract. An isolated composition can, however, also be substantially pure. An isolated composition can be in a homogeneous state and can be in a dry or an aqueous solution. Purity and homogeneity can be determined, for example, using analytical chemistry techniques such as polyacrylamide gel electrophoresis (SDS-PAGE) or high performance liquid chromatography (HPLC).

The term "nucleic acid" or "nucleic acid sequence" refers to a deoxyribonucleotide or ribonucleotide oligonucleotide in either single- or double-stranded form. The term encompasses nucleic acids, *i.e.*, oligonucleotides, containing known analogues

of natural nucleotides which have similar or improved binding properties, for the purposes desired, as the reference nucleic acid. The term also includes nucleic acids which are metabolized in a manner similar to naturally occurring nucleotides or at rates that are improved thereover for the purposes desired. The term also encompasses nucleic-acid-like structures with synthetic backbones. DNA backbone analogues provided by the invention include phosphodiester, phosphorothioate, phosphorodithioate, methylphosphonate, phosphoramidate, alkyl phosphotriester, sulfamate, 3'-thioacetal, methylene(methylimino), 3'-N-carbamate, morpholino carbamate, and peptide nucleic acids (PNAs); see Oligonucleotides and Analogues, a Practical Approach, edited by F. Eckstein, IRL Press at Oxford University Press (1991); Antisense Strategies, Annals of the New York Academy of Sciences, Volume 600, Eds. Baserga and Denhardt (NYAS 1992); Milligan (1993) *J. Med. Chem.* 36:1923-1937; Antisense Research and Applications (1993, CRC Press). PNAs contain non-ionic backbones, such as N-(2-aminoethyl) glycine units. Phosphorothioate linkages are described in WO 97/03211; WO 96/39154; Mata (1997) *Toxicol Appl Pharmacol* 144:189-197. Other synthetic backbones encompasses by the term include methyl-phosphonate linkages or alternating methylphosphonate and phosphodiester linkages (Strauss-Soukup (1997) *Biochemistry* 36:8692-8698), and benzylphosphonate linkages (Samstag (1996) *Antisense Nucleic Acid Drug Dev* 6:153-156). The term nucleic acid is used interchangeably with gene, cDNA, mRNA, oligonucleotide primer, probe and amplification product.

The term "pharmaceutical composition" refers to a composition suitable for pharmaceutical use in a subject. The pharmaceutical compositions of this invention are formulations that comprise a pharmacologically effective amount of an MBP peptide and/or a Class II:peptide complex and a pharmaceutically acceptable carrier.

The terms "to treat" and "treatment" refer to prophylactic and therapeutic treatment administered to a subject. Prophylactic treatment includes administering treatment to an individual who does not have a disease or condition, or does not exhibit signs of a disease or condition, or exhibits only early signs of a disease or condition for the purpose of decreasing or abrogating the risk of acquiring the disease or condition or decreasing or abrogating a symptom or a pathologic condition arising from or related to the disease or pathology. The invention provides a method to treat a T cell mediated immune response

against myelin basic protein in a subject by administration of an MBP peptide and/or a Class II:peptide complex to an individual who is considered a high risk for developing a demyelinating disease or condition. Therapeutic treatment also includes administering treatment to an individual who exhibits signs of pathology or disease, or is considered at risk for developing or incurring such a condition or disease, for the purpose of preventing, diminishing, ameliorating or eliminating any symptoms or pathology. Thus, the methods of the invention provide a means to treat demyelinating disease and to prevent, diminish, eliminate or otherwise ameliorate the disease's clinical symptoms and tissue pathology, as discussed above. A "pharmacologically effective amount" is the amount of compound administered to the individual to prophylactically or therapeutically effectively treat an individual for a disease or condition.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

EXAMPLES

The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1 **Identification of core structure and critical TCR contact residues in an antigenic MBP peptide by measuring T cell activation signals**

The invention provides novel MBP peptides and MPB peptide:Class II complexes, pharmaceutical formulations comprising these compositions, and a method for treating a T cell mediated immune response against MPB in a subject by administration of these novel compositions. The following example details the identification of novel MBP peptides which include the core TCR recognition sequence amino acid residues involved in the binding of autoreactive TCR associated with demyelinating disease, as MS, with MBP peptide and an appropriate Class II molecule.

A novel, minimal structure and critical residues for its interaction with autoreactive TCR was identified for human myelin basic protein (MBP) peptide. Synthetic

peptide analogs were incubated with TCRs on a DRB5*0101-restricted transformed human T cell clone (SS8T) expressing MHC class II (DR2) molecules. A silicon-based biosensor microphysiometer was used to measure the real time cell response to a T cell activation event stimulated by the binding of Class II:peptide to TCR. The microphysiometer monitored changes in extracellular acidification rate in response to MBP peptide:Class II polypeptide binding to autoreactive TCRs. Increases in the extracellular acidification rate is a direct result of early T cell signaling events, such as high affinity TCR binding to a Class II:peptide complex. Cultured SS8T cells were exposed to N- (amino)-terminus and C- (carboxy)-terminus truncated MBP peptides separately in microphysiometer chambers to determine the minimal amino acid residues required for the T cell response. In parallel, analogs of MBP peptide with single alanine substitutions were tested in this assay to identify critical amino acid residues involved in TCR interactions. As described below, a minimal core length of ten amino acid residues was determined to correspond to peptide MBP(91-100) (SEQ ID NO:38) and residues F-91, K-93, N-94, I-95 and V-96 were determined to be essential for TCR interaction. Acidification rate measurements correlated well with enhanced levels of gamma-interferon (IFN) and tumor necrosis factor (TNF)-alpha cytokine production.

Herpes virus saimiri (HVS) immortalized human T cells were used in *in vitro* assays to identify the novel MBP peptides of the invention. HVS T cells express the same level of TCR as normal human T cells, without any loss of antigen recognition (Weber (1993) *Proc. Natl. Acad. Sci. USA* 90:11049). Specifically, the HVS transformed human T cell clone used in these assays, designated SS8T, was generated from an MS patient. SS8T has previously been characterized as being restricted to the HLA Class II DR2(DRB5*0101) polypeptide and the MBP(84-102) peptide (Weber (1993) *supra*). A modified peptide analog of MBP(84-102) with a N-acetylated tyrosine residue at the N-terminus (Ac-MBP(83-102)Y^{83}) has been characterized as being recognized by SS8T transformed T cells when complexed with HLA DR2 (DRB5*0101) (Mukku (1995) *Mol. Immunol.* 32:555; Arimilli (1995) *J. Biol. Chem.* 270:971). To identify the minimum length and critical TCR contact residues of this MBP(83-102)Y^{83} peptide, different terminal truncated and alanine analog peptides were synthesized. The various peptides used in this study are presented schematically in Fig. 1A and Fig 1B. For alanine analog peptides, a single amino acid was substituted with alanine for each residue in the MBP(90-102)Y^{83} sequence.

The critical MBP peptide amino acid residues required for TCR engagement in this model system depend on two factors; the peptide residues responsible for binding to MHC class II binding groove amino acid residues (the peptide agretope) and the effective interaction of some of the amino acid residues of the peptide with TCR (the epitope). To determine the minimum length of the immunodominant epitope, various terminally truncated peptides were incubated at different concentrations with SS8T cells in a microtiter plate for 48 hours at 37°C. After incubating the T cells with peptide, culture fluids were tested for the presence of secreted gamma IFN and TNF-beta by ELISA using monoclonal antibodies (mAbs) to each (anti-human gamma IFN mAb and rabbit anti-human gamma IFN polyclonal Ab, Endogen Inc., Woburn, MA; peroxidase-conjugated goat IgG and rabbit IgG, Jackson Immunoresearch Laboratories, West Grove, PA; human gamma IFN, Boehringer Mannheim, Indianapolis, IN; anti-human TNF-beta mAb, goat anti-human TNF-beta polyclonal and recombinant human TNF-beta, R&D Systems Inc, Minneapolis, MN). In parallel analyses, SS8T cells were immobilized in agarose and exposed to various terminal truncated peptides in the microphysiometer chamber to monitor their extracellular acidification rates.

Peptides prepared for these studies were acetylated at the N (amino)-terminus and amidated at the C (carboxy)-terminus. The MBP(83-102) peptide with the sequence Ac-YDENPVVHFFKNIVTPRTPP (SEQ ID NO:1); and the MBP(124-143) peptide with the sequence Ac-GFGYGGRASDYKSAHKGFKG (SEQ ID NO:37) were synthesized by the standard solid phase method using side-chain protected Fmoc amino acids on an Applied Biosystems 431A automated peptide synthesizer. The deprotected, crude peptides were purified by reverse-phase HPLC, and the homogeneity and identity of the purified peptides were confirmed by mass spectrometry. All terminally truncated and alanine analog MBP peptides (Fig. 1) were synthesized by solid phase peptide synthesis using Fmoc chemistry. All chemicals, including the Rink amide MBRA resin and side chain protected Fmoc amino acids were obtained from Nova Biochem, San Diego, CA. HBTU/HOBt or PyBOP activation chemistry were employed for protected amino acid coupling on ABI 431 A automated peptide synthesizer or ABIMED/GILSON AMS 422 multiple peptide synthesizer, as described by Luu (1996) *Int. J. Peptide Protein Res.* 47:91. After peptides were synthesized on solid phase, they were cleaved by TFA containing 5% 4-methoxybenzenethiol and 5% 4-methylmercaptophenol as scavengers. The crude peptides were precipitated by pentane:acetone mixture (4:1, v:v) and

isolated by centrifugation. Peptides were washed with pentane:acetone mixture three times followed by pentane and were dried in vacuum. Peptides were purified by reverse phase HPLC using a C18 column; the pure fractions were pooled and lyophilized. Peptides were characterized by electron spray mass spectrometry.

T cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 ug/ml streptomycin, 10% fetal bovine serum and 50 units/ml human recombinant IL-2 (rIL-2) at 37°C. Every alternate day, cells were transferred to fresh culture medium. Various peptides at different concentrations were incubated with the cells in a microtiter tissue culture plate at a density of 20,000 cells/200 ul/well in the absence of rIL-2. After 48 h of incubation at 37°C, the culture fluids were collected from each well to test for gamma-IFN and TNF-beta cytokines. The detection of gamma-IFN was by Ab ELISA as described by Arimilli (1995) *supra*. For the detection of TNF-beta, Nunc Maxisorb 96-well plates were coated with anti-human TNF-beta mAb at a concentration of 0.5 ug/well and incubated at 4°C overnight. The wells were blocked with 0.1% bovine serum albumin, and samples were incubated at RT for 2 h. A standard curve was generated by using recombinant human TNF-beta with a dilution range of 500 to 0.01 ng/ml. Goat anti-human TNF-beta was then added at a concentration of 1 ug/ml and plates were incubated at 25°C for an additional 2 h. Wells were washed three times and incubated with HRP-conjugated mouse anti-goat Ab at a concentration of 1 ug/ml for 1 h at 21°C, prior to developing color using 3,3',5,5'-tetramethyl benzidine (TMB, Moss, Inc, Pasadena, MD) as a substrate. The reaction was stopped by 2N sulfuric acid at 5 min, and the absorbance was measured at 450 nm.

Freshly cultured SS8T cells were immobilized into microphysiometer cell capsules as described by Nag et al., 1992, using low-melt agarose (Molecular Devices Corp., Sunnyvale, CA). Briefly, T cells were rested from IL-2 pulsing for 2 days. Cells were counted and suspended in a serum-free loading medium (low-buffering RPMI 1640 containing 10% fatty acid free, endotoxin-free, BSA). Cells were collected by centrifugation and resuspended at a concentration of 3 x 10^5 T cells per 7.5 ul of loading medium. Low-melt agarose, melted and stored at 37°C, was added to the suspended cells to a concentration of 2.5 ul per 7.5 ul. Ten ul of the agarose/cell mixture was immediately spotted into the center of the cell capsule cups (Molecular Devices Corp.) held in a 12 well culture plate. After 5 minutes 2 ml of loading medium was placed in the capsule cup over the solidified agarose and

a membrane insert was placed over the cells. The assembled cell capsule was loaded in the Cytosensor chamber at 37°C and perfused at 50 ul per min with low-buffering RPMI 1640 medium containing 10% of BSA per ml but no added HEPES or bicarbonate. Extracellular acidification measurements were made in the Cytosensor microphysiometer as described by McConnell (1992) *Science* 257:1906, by collecting potentiometric measurements for 45 seconds every 2 min. Acidification rate data (uV/sec) were normalized to 100% prior to cell stimulation, which allowed for comparison of data from cells in separate chambers.

Figure 2A, 2B and 2C show the results of these analyses, demonstrating increased levels of gamma-IFN, TNF-beta and extracellular acidification rates with first six N-terminal truncated peptides: MBP(84-102) (SEQ ID NO:2), MBP(85-102) (SEQ ID NO:3), MBP(86-102) (SEQ ID NO:4), MBP(87-102) (SEQ ID NO:5), MBP(88-102) (SEQ ID NO:6), and MBP(89-102) (SEQ ID NO:7). The SS8T cells exposed to these peptides show a 2 to 4 fold increase in gamma IFN and a 3 to 4 fold increase in TNF-beta production in a dose dependent manner (Fig. 2A, 2B). Similarly, increase in acidification rates at 118% of basal rate were observed within 10 min (Fig. 2C), indicating that the amino acid residue 84, residue 85, residue 86, residue 87, residue 88, and residue 89 are not important for TCR recognition in SS8T cells. The gamma-IFN and TNF-beta cytokine concentrations measured after 48 hour (h) correlated well with the acidification rates.

Figure 2D, 2E and 2F show results comparing the gamma-IFN, TNF-beta and acidification rates of next five N-terminus truncated MBP(90-102) (SEQ ID NO:8), MBP(91-102) (SEQ ID NO:9), MBP(92-102) (SEQ ID NO:10), MBP(93-102) (SEQ ID NO:11), and MBP(94-102) (SEQ ID NO:12) peptides. Among them the truncation of amino acid residue 91, residue 92, and residue 93 did not produce gamma-IFN, TNF-beta cytokines and acidification rates. This demonstrates that these residues are critical in T cell stimulation. Since, in one study, residue 92 (F-92) was suggested to be important for binding to Class II (Wucherpfennig (1994) *J. Exp. Med.* 179:279). The absence of T cell response by Class II:MBP(93-102) (SEQ ID NO:11) complex could be due to lack of peptide binding to class II polypeptide or lack of critical TCR contact residues (epitope residues).

In a separate assay, SS8T cells were incubated with six C-terminal truncated peptides MBP(83-101) (SEQ ID NO:13), MBP(83-100) (SEQ ID NO:14), MBP(83-99) (SEQ ID NO:15), MBP(83-98) (SEQ ID NO:16), MBP(83-97) (SEQ ID NO:17) and MBP(83-96)

(SEQ ID NO:18). As shown in Fig. 3A, 3B, and 3C, only the parent sequence (SEQ ID NO:1) and MBP(83-101) (SEQ ID NO:13) showed an increase in gamma-IFN, TNF-beta or acidification rates. This demonstrates that that C-terminal amino acid residue 100, residue 99, residue 98, residue 97 and residue 96 are important in TCR stimulation by MBP. Small quantities of gamma-IFN and TNF-beta were produced with MBP(83-100) (SEQ ID NO:14) and MBP(83-99) (SEQ ID NO:15) peptides. Similar analysis of the additional five C-terminal truncated peptides MBP(83-95) (SEQ ID NO:19), MBP(83-94) (SEQ ID NO:20), MBP(83-93) (SEQ ID NO:21), MBP(83-92) (SEQ ID NO:22), and MBP(83-91) (SEQ ID NO:23), did not show either stimulation of gamma-IFN, TNF-beta or an increase in acidification rate (Fig. 3D, 3E and 3F). These data demonstrate that amino acid residue 92, residue 93, residue 94 and residue 95 are important in T cell recognition of the peptide. The results of both N-terminal and C-terminal truncation demonstrate that FFKNIVTPRT (MBP(91-100) (SEQ ID NO:38)) is the minimal core sequence for TCR stimulation by MBP when presented by Class II DRB5*0101.

The contribution of individual amino acids in a peptide towards TCR engagement can be studied by various methods, including the D-amino acid analogs, substitution of conserved amino acid residues, switching of charge on the amino acids, and alanine substitution. Alanine substitution is more popular since it is the simplest side chain amino acid with a chiral center, and it creates only minor disturbances in the secondary structure of polypeptides and proteins. It is a common replacement for all but aromatic amino acids, based on comparison of evolutionary related proteins. Therefore, an alanine substitution strategy was used to define important TCR contact residues. Various analog peptides from MBP(90-102) (SEQ ID NO:8) with single amino acid substitutions using alanine were incubated with SS8T cells. The culture fluids were tested for gamma-IFN, TNF-beta and compared with acidification rates. Figures 4A, 4B and 4C show that two peptides, MBP(83-102)A90 (SEQ ID NO:24) and MBP(83-102)A92 (SEQ ID NO:26), were able to induce gamma-IFN, TNF-beta along with increased acidification rates when presented to SS8T cells by Class II DRB5*0101. MBP(83-102)A91 (SEQ ID NO:25), MDP(83-102)A93 (SEQ ID NO:27), MDP(83-102)A94 (SEQ ID NO:28), and MDP(83-102)A95 (SEQ ID NO:29), peptides failed to induce cytokines and showed no increase in acidification rates. This demonstrates that the amino acid residues F-91, K-93, N-94, and I-95 are important in

T cell stimulation. In one study, residue F-91 and K-93 were suggested to be secondary and N-94 to be the primary TCR contact residues in the MBP(84-102) peptide (Vergelli (1997) *J. Immunol.* 158:3746). The instant data demonstrates that residues F-91, K-93, N-94, and I-95 are important TCR contact residues in the MBP(84-102) peptide in this system.

The alanine analogs MBP(83-102)A97 (SEQ ID NO:31), MBP(83-102)A98 (SEQ ID NO:32), MBP(83-102)A99 (SEQ ID NO:33), MBP(83-102)A100 (SEQ ID NO:34), MBP(83-102)A101 (SEQ ID NO:35), and MBP(83-102)A102 (SEQ ID NO:36) show increasing levels of gamma-IFN, TNF-beta cytokines along with increased acidification rates. Only MBP(83-102)A96 (SEQ ID NO:30) failed to induce cytokines and showed no increase in acidification rates (Fig. 4D, 4E and 4F). This experiment demonstrates that V-96 is also an important residue for T cell recognition. As shown in Figure 4F, except for the peptide MDP(83-102)A96 (SEQ ID NO:30), all peptides show an immediate increase in acidification rate within 10 min. Thus, the acidification rates with alanine analog peptides was correlated with the gamma-IFN and TNF-beta production by SS8T cells. In another study, the residues N-94 and V96 were suggested to be TCR contact residues for a DRB5*0101 and MBP(84-102) peptide restricted T cell clone (Wucherpfennig (1994) *supra*).

In all experiments, MBP(124-143) (SEQ ID NO:37) was used as a negative control. MBP(124-143) peptide has a high affinity for binding to DR2 (Mukku (1995) *supra*), but does not stimulate DRB5*0101 restricted T cells (Arimilli (1995) *supra*).

Figure 5A highlights (boxed area) the core TCR recognition sequence as MBP(91-100) (SEQ ID NO:38). As described above, this peptide was identified by measuring extracellular acidification rates and by the gamma-IFN, TNF-beta cytokine response in SS8T cells using terminally truncated peptides. Figure 5B indicates (by arrows) the important amino acid residues involved in TCR contact as F-91, K-93, N-94, I-95, and V-96. As described above, these amino acids were identified from extracellular acidification rates and gamma-IFN, TNF-beta cytokine response in SS8T cells by using alanine analog peptides.

Studies were performed to demonstrate a requirement for the peptides to bind Class II polypeptide prior to T cell stimulation, *i.e.*, to determine whether T cell stimulation is the direct consequence of TCR engagement through MHC-peptide complex formed on the cell surface or due to the direct binding of the peptide to the TCR. Ab blocking experiments were performed using anti-TCR mAb and anti-Class II mAb (hybridoma cell line L243,

producing mAb to monomorphic human HLA DR molecules was obtained from American Type Culture Collection, Bethesda, MD). Transformed SS8T human T cells bear Class II on their surface, confirmed by flow cytometry. SS8T cells were incubated with either anti-TCR or anti-DR Ab to block the TCR and Class II sites. The cells were then washed and loaded in the microphysiometer chambers and exposed to 10 ug/ml MBP(83-102) (SEQ ID NO:1) peptide. Blocking TCR or Class II with specific mAb resulted in complete inhibition in acidification rate increase. These results also demonstrate that peptide binding to Class II molecules on a cell surface is a rapid cell activation phenomenon (McConnell (1995) *Proc. Natl. Acad. Sci. USA* 92:2750). After the high affinity binding of TCR with peptide:Class II polypeptide complex, early cell activation events (signals), as measured by extracellular acidification by microphysiometer, occur within 10 min. Later cell activation events (signals) are measured within 48 hours; representative later activation events measured in these studies were the gamma-IFN and TNF-beta cytokine response. Measurement of early and late activation events are equivalent in identifying peptides that are important for TCR recognition, as was done to identify the novel MBP peptides of the invention.

In summary, these studies have identified a novel family of MBP peptides characterized by having an amino acid sequence Phe -X - Lys - Asn - Ile - Val - X - X - X - Thr - X -X, wherein X is any amino acid. These peptides are also included in novel Class II:MBP complexes of the invention. These complexes comprise an MHC Class II complex capable of binding a T cell receptor, the complex consisting essentially of: an MHC Class II polypeptide comprising an extracellular domain of an MHC Class II molecule sufficient to form an antigen binding pocket and a myelin basic protein having an amino acid sequence Phe -X - Lys - R_1 - Ile - Val - X - X - X - Thr - X -X , wherein X is any amino acid, and R_1 is Asn or Gln; wherein the MBP peptide is bound to the MHC Class II component antigen binding pocket.

WHAT IS CLAIMED IS:

1. An isolated myelin basic protein (MBP) peptide, said peptide characterized by having an amino acid sequence Phe -X - Lys - Asn - Ile - Val - X - X - X - Thr - X -X, wherein X is any amino acid.

2. The isolated myelin basic protein peptide of claim 1, said peptide characterized by having an amino acid sequence Phe - Phe - Lys - Asn - Ile - Val - Thr - Pro - Arg - Thr - Pro - Pro, or a conservative substitution thereof.

3. The isolated myelin basic protein peptide of claim 1, which is linked to a heterologous sequence.

4. The isolated myelin basic protein peptide of claim 3, which is a fusion protein.

5. An isolated myelin basic protein peptide which specifically binds to an antibody directed against a peptide characterized by having an amino acid sequence Phe - Phe - Lys - Asn - Ile - Val - Thr - Pro - Arg - Thr - Pro - Pro.

6. An isolated nucleic acid encoding a myelin basic protein peptide, said peptide characterized by having an amino acid sequence Phe - X - Lys - Asn - Ile - Val - X - X - X - Thr - X - X , wherein X is any amino acid.

7. The isolated nucleic acid of claim 6, wherein the encoded myelin basic protein peptide is characterized by having an amino acid sequence Phe - Phe - Lys - Asn - Ile - Val - Thr - Pro - Arg - Thr - Pro - Pro , or a conservative substitution thereof.

8. The isolated nucleic acid of claim 6, wherein the nucleic acid comprises SEQ ID NO:1.

9. A composition comprising an MHC Class II complex capable of binding a T cell receptor, the complex consisting essentially of:

an MHC Class II polypeptide comprising an extracellular domain of an MHC Class II molecule sufficient to form an antigen binding pocket, said component being encoded by an allele associated with an autoimmune disease directed to myelin basic protein, wherein the Class II component is soluble under physiological conditions in the absence of detergent or lipid; and,

a myelin basic protein having an amino acid sequence

Phe -X - Lys - R_1 - Ile - Val - X - X - X - Thr - X -X , wherein X is any amino acid, and R_1 is Asn or Gln;

wherein the myelin basic protein peptide is bound to the MHC Class II component antigen binding pocket.

10. The composition of claim 9, wherein the myelin basic protein peptide has an amino acid sequence Phe - Phe - Lys - Asn - Ile - Val - Thr - Pro - Arg - Thr - Pro - Pro or a conservative substitution thereof.

11. The composition of claim 9, which is a fusion protein.

12. The composition of claim 9, further comprising an effector composition.

13. The composition of claim 9, wherein the autoimmune disease directed to myelin basic protein is multiple sclerosis.

14. The composition of claim 9, wherein the Class II polypeptide comprises the antigen binding pocket of an HLA DR2.

15. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a pharmacologically effective amount of a peptide characterized by having an amino acid sequence Phe -X - Lys - Asn - Ile - Val - X - X - X - Thr - X -X, wherein X is any amino acid.

16. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a pharmacologically effective amount of the composition of claim 9.

17. An antibody, specifically immunoreactive under immunologically reactive conditions, to a myelin basic protein peptide, said peptide characterized by having an amino acid sequence Phe - Phe - Lys - Asn - Ile - Val - Thr - Pro - Arg - Thr - Pro - Pro.

18. An antibody, specifically immunoreactive under immunologically reactive conditions, to a myelin basic protein peptide comprising the peptide encoded by the nucleic acid of claim 6.

19. A method for inhibiting a T cell mediated immune response against myelin basic protein in a subject, comprising administering to the subject the peptide of claim 1 or the composition of claim 9 in an amount effective to treat the T cell mediated immune response.

20. The method of claim 17, wherein the T cell mediated immune response causes a pathology to a neurological system.

21. The method of claim 17, wherein the pathology to the neurological system is characterized as multiple sclerosis.

22. A method of identifying a T cell epitope on an antigen which, when bound to the antigen binding pocket of an MHC Class II molecule, is capable of binding to a T cell receptor, such binding triggering an extracellular acidification reaction by a T cell expressing the T cell receptor, said method comprising the steps of:

a) providing a composition comprising the T cell epitope bound to the antigen binding pocket of an MHC Class II molecule;

b) contacting a T cell expressing the T cell receptor with the epitope; and

c) measuring the extracellular acidification, wherein a change in the extracellular acidification indicates the binding of T cell epitope to the T cell receptor.

23. The method of claim 20, wherein the change in extracellular acidification is measured using a microphysiometer.

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FIGUR

1

1 A

Truncated peptides from Ac-MBP(83-102)Y83

MBP(83-102)	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
MBP(84-102)		Ac-D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
MBP(85-102)			Ac-E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
MBP(86-102)				Ac-N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
MBP(87-102)					Ac-P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
MBP(88-102)						Ac-V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
MBP(89-102)							Ac-V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
MBP(90-102)								Ac-H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
MBP(91-102)									Ac-F	F	K	N	I	V	T	P	R	T	P	P	NH_2
MBP(92-102)										Ac-F	K	N	I	V	T	P	R	T	P	P	NH_2
MBP(93-102)											Ac-K	N	I	V	T	P	R	T	P	P	NH_2
MBP(94-102)												Ac-N	I	V	T	P	R	T	P	P	NH_2

MBP(83-102)	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
MBP(83-101)	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	NH_2	
MBP(83-100)	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	NH_2		
MBP (83-99)	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	NH_2			
MBP (83-98)	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	NH_2				
MBP (83-97)	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	NH_2					
MBP (83-96)	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	NH_2						
MBP (83-95)	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	NH_2							
MBP (83-94)	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	NH_2								
MBP (83-93)	Ac-Y	D	E	N	P	V	V	H	F	F	K	NH_2									
MBP (83-92)	Ac-Y	D	E	N	P	V	V	H	F	F	NH_2										
MBP (83-91)	Ac-Y	D	E	N	P	V	V	H	F	NH_2											

1 B

Ac-MBP (83-102)Y83 alanine substituted peptides

Parent	Sequence	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	
	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P-NH_2		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
84	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2	
85	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2	
86	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2	
87	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2	
88	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2	
89	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2	
A90	Ac-Y	D	E	N	P	V	V	A	F	F	K	N	I	V	T	P	R	T	P	P	NH_2	
A91	Ac-Y	D	E	N	P	V	V	H	A	F	K	N	I	V	T	P	R	T	P	P	NH_2	
A92	Ac-Y	D	E	N	P	V	V	H	F	A	K	N	I	V	T	P	R	T	P	P	NH_2	
A93	Ac-Y	D	E	N	P	V	V	H	F	F	A	N	I	V	T	P	R	T	P	P	NH_2	
A94	Ac-Y	D	E	N	P	V	V	H	F	F	K	A	I	V	T	P	R	T	P	P	NH_2	
A95	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	A	V	T	P	R	T	P	P	NH_2	
A96	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	A	T	P	R	T	P	P	NH_2	
A97	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	A	P	R	T	P	P	NH_2	
A98	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	A	R	T	P	P	NH_2	
A99	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	A	T	P	P	NH_2	
A100	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	A	P	P	NH_2	
A101	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	A	P	NH_2	
A102	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	A	NH_2	
		83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	

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FIGURE 2

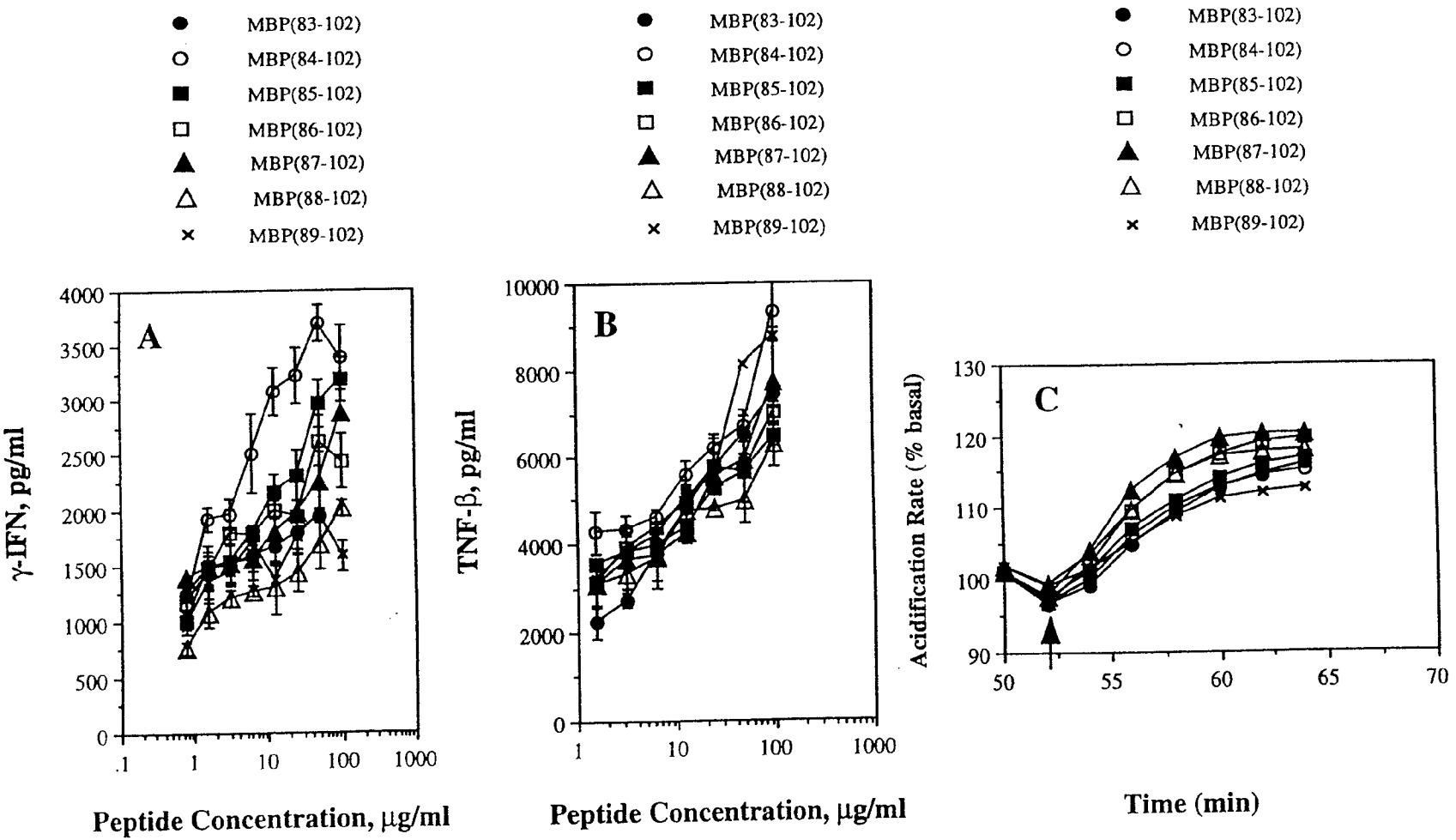

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FIGURE 2

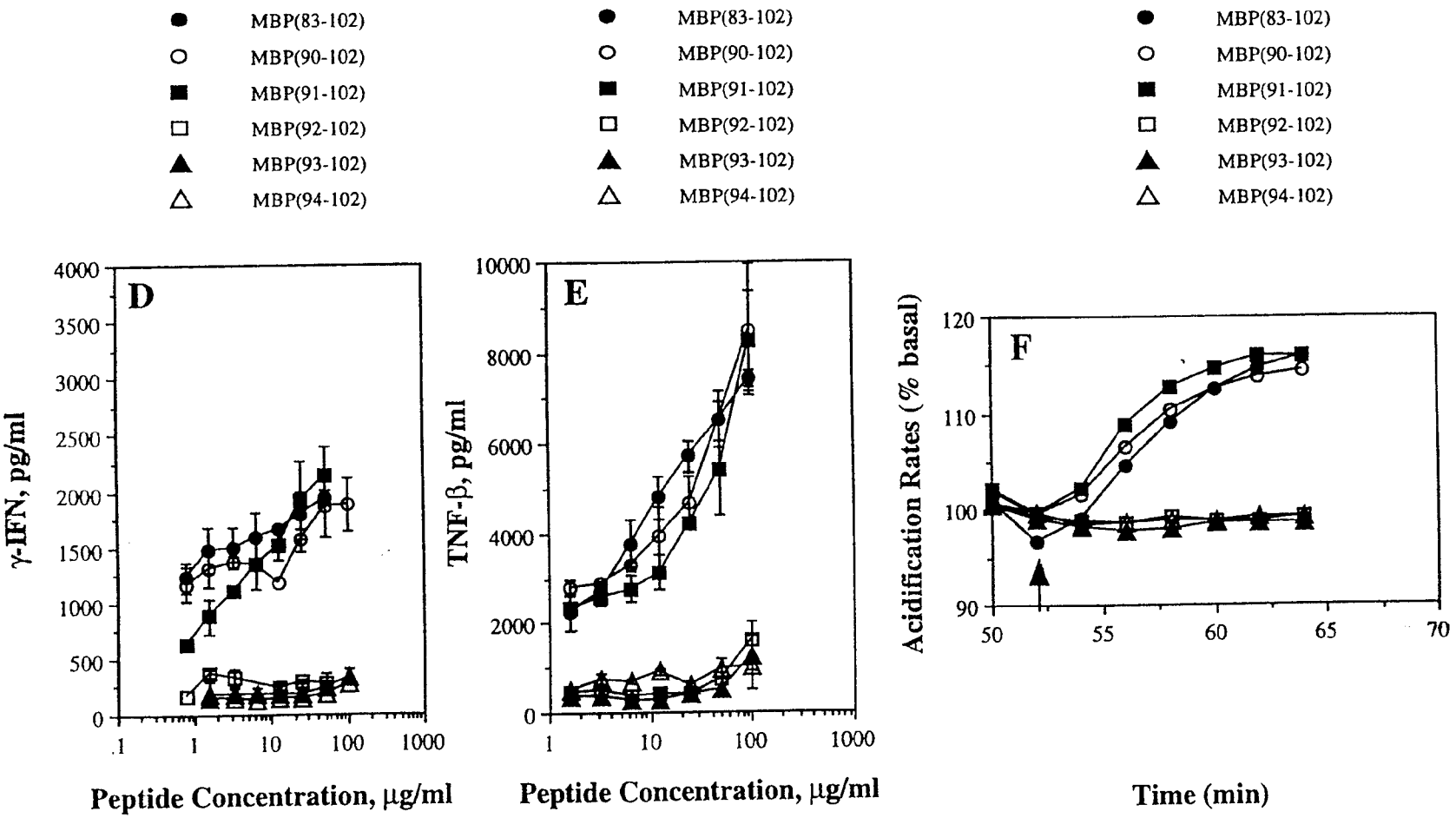

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FIGURE 3

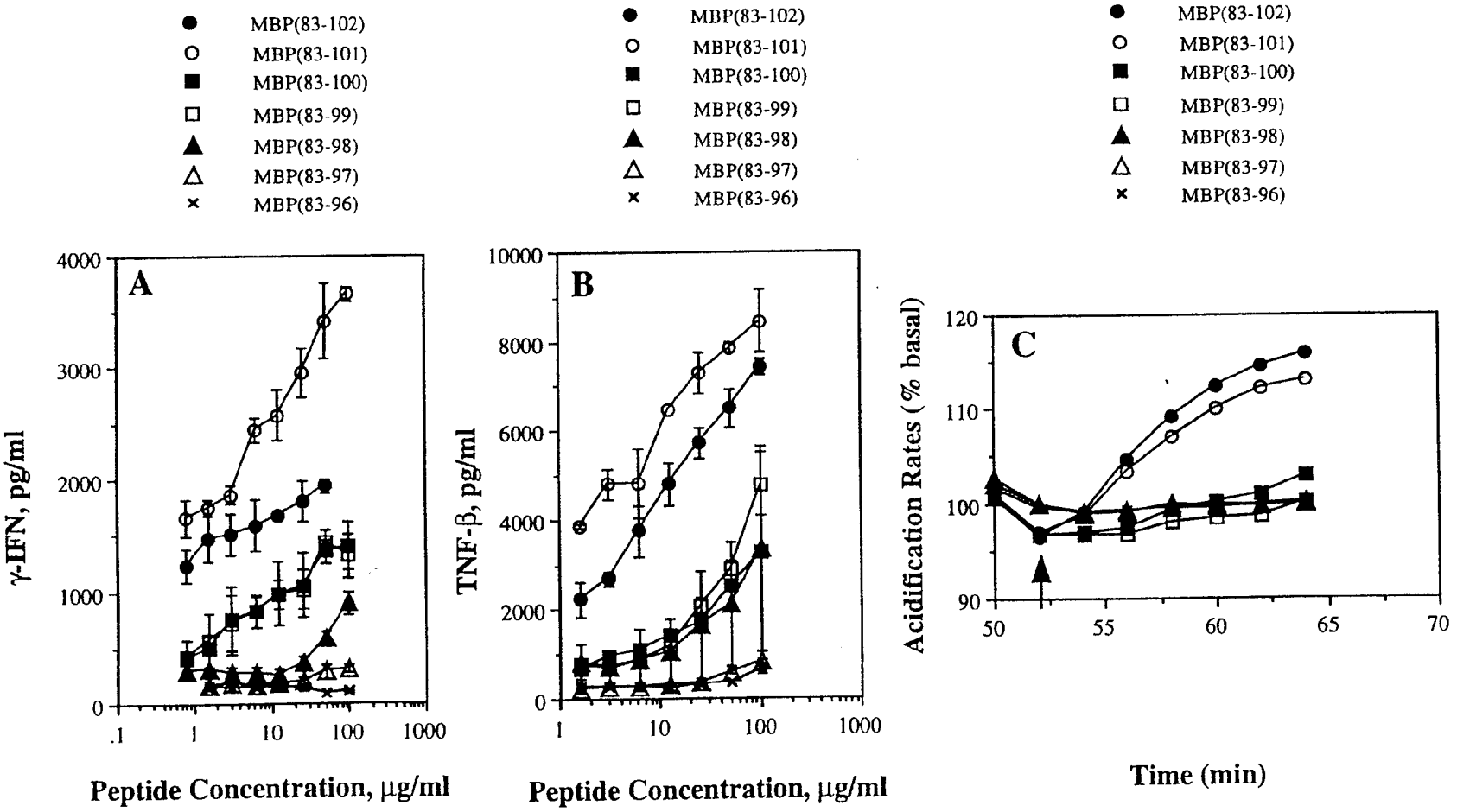

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FIGURE 3

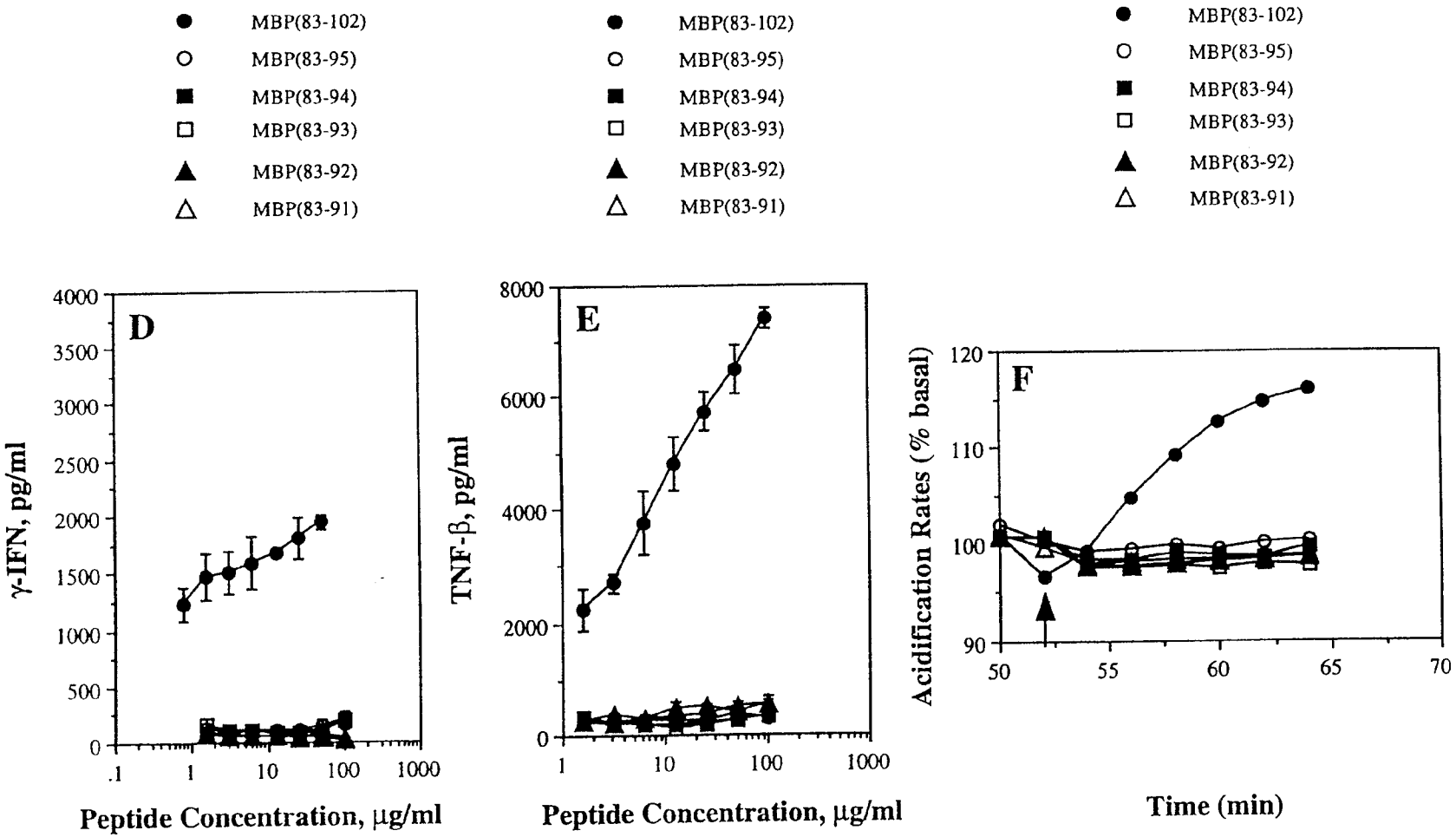

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FIGURE 4

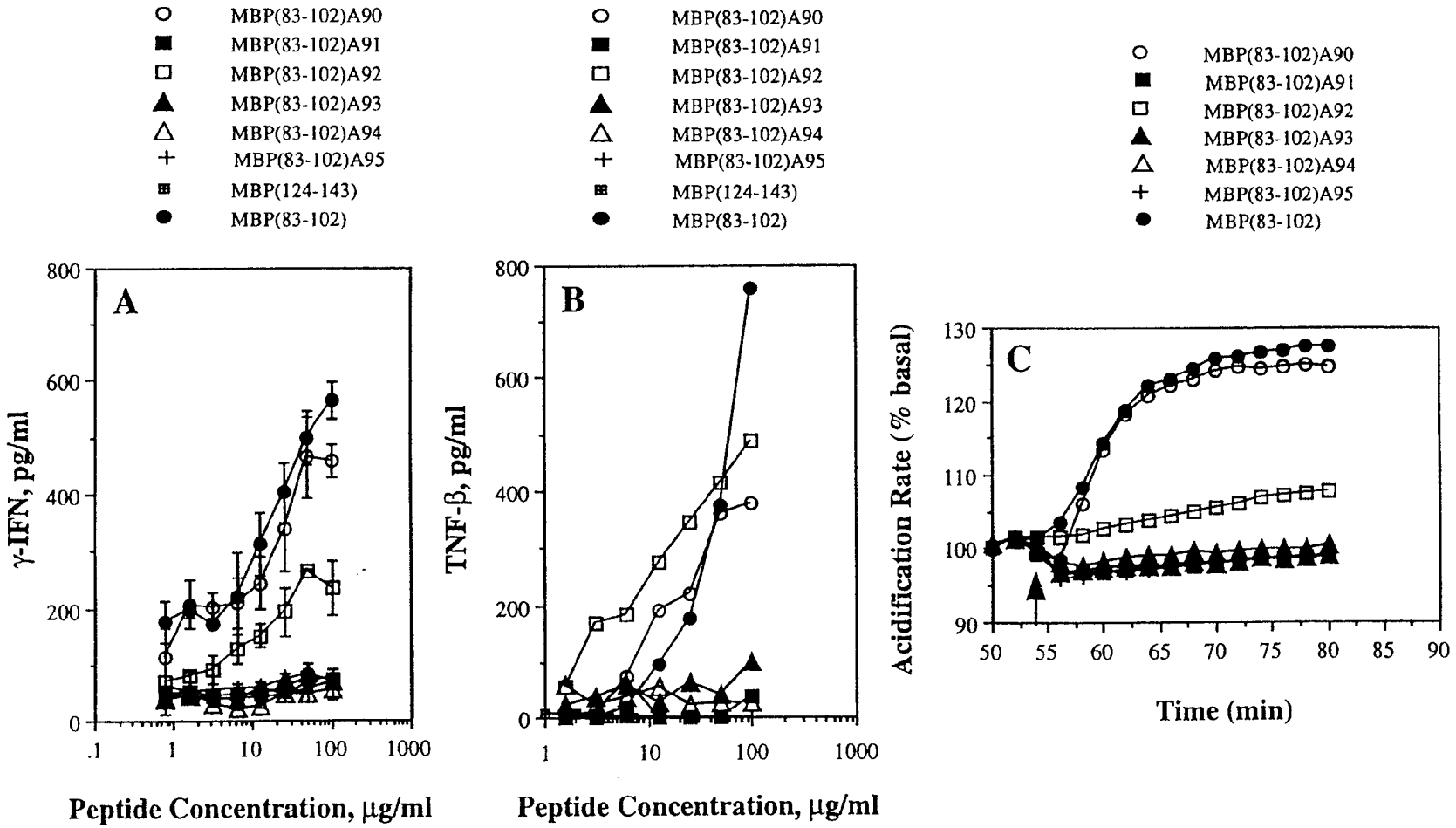

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FIGURE 4

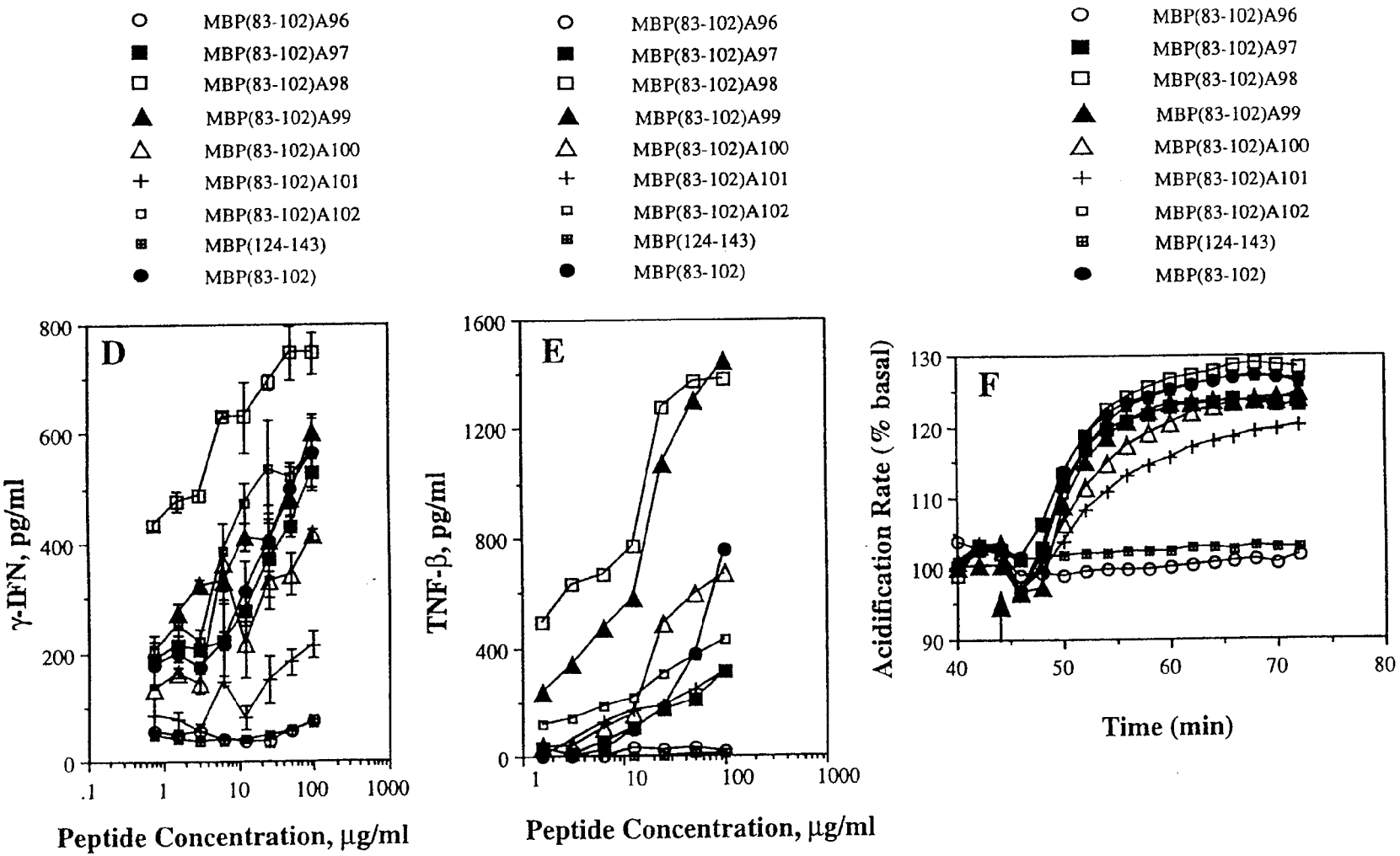

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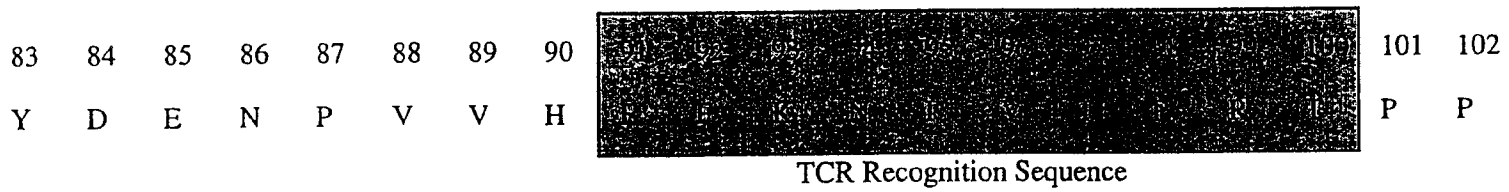
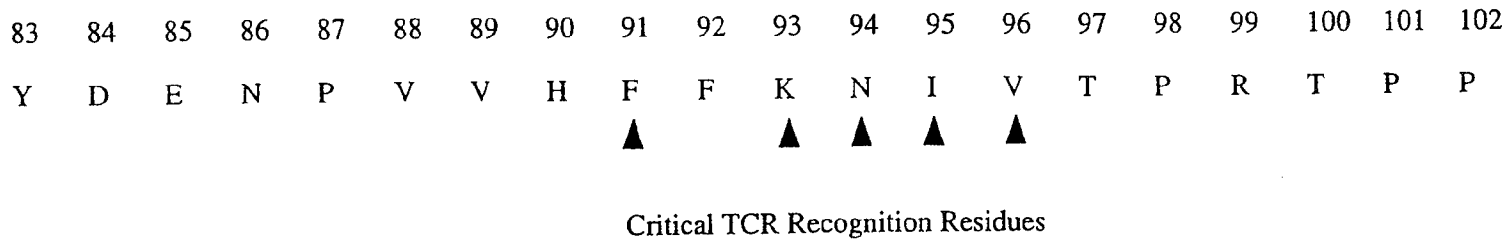
FIGURE 5

A. Terminal Truncation



B. Alanine Analogs



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(54) Title: MYELIN BASIC PROTEIN PEPTIDES AND USES THEREOF

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A. CLASSIFICATION OF SUBJECT MATTER

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, MEDLINE, SCISEARCH, DERWENT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,734,023 A (NAG et al.) 31 March 1998, see entire article.	1-23
Y	WO 96/40944 A2 (ZYMOGENETICS, INC.) 19 December 1996, see entire article.	1-23
Y	NAG et al. Functionally Active Recombinant α and β Chain-Peptide Complexes Of Human Major Histocompatibility Class II Molecules. The Journal of biological chemistry. April 1996, Vol. 271, No. 17, pages 10413-10418, see entire article.	1-23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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This invention pertains generally to the treatment of autoimmune diseases of the central nervous system characterized by demyelination. In particular, this invention pertains to novel peptides derived from human myelin basic protein (MBP). When complexed with the appropriate major histocompatibility complex (MHC) molecule, these peptides can be used to treat multiple sclerosis and other demyelinating autoimmune diseases.

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MYELIN BASIC PROTEIN PEPTIDES AND USES THEREOF

FIELD OF THE INVENTION

This invention pertains generally to the treatment of autoimmune diseases of the central nervous system characterized by demyelination. In particular, this invention pertains to novel peptides derived from human myelin basic protein. These peptides can be used as pharmaceuticals to treat multiple sclerosis and other demyelinating autoimmune diseases alone or complexed with the appropriate major histocompatibility complex (MHC) molecule.

BACKGROUND OF THE INVENTION

Multiple sclerosis (MS) is a chronic, inflammatory, autoimmune disease of the human central nervous system (CNS) characterized by demyelination and by local infiltrates of macrophages, plasma cells and T cells in the CNS (Allen (1991) *Pathology of Multiple Sclerosis*, p341 in *McAlpine's Multiple Sclerosis*; Matthewse, *et al.*, eds, Churchill Livingstone, Edinburgh). MS is an autoimmune disease directed at myelin and the myelin-producing oligodendrocyte. In MS and other demylelinating diseases, T cell clones specific for a component of myelin, myelin basic protein (MBP), cause the demyelination of nerve sheaths in the CNS. In addition to MBP-restricted T lymphocyte clones, demyelinating inflammatory lesions can contain multiple nonrestricted immune cells capable of mediating tissue injury (Zamvil (1990) *Ann. Rev. Immunol.* 8:579-621; Wucherpfenning (1991) *Immunol. Today* 12:277-282; Martin (1992) *J. Immunol.* 148:1359-1366; Martin (1992) *Ann. Rev. Immunol.* 10:153-187).

Major histocompatibility complex (MHC) class II (DR) molecules are heterodimers displayed on the cell surface of antigen processing/ presenting cells (APCs). In demyelinating disease, APC Class II molecules "present" MBP self-peptides derived from myelin and oligodendrocytes to helper (CD4+) T lymphocytes. The Class II molecules literally present the peptide to the T cell by binding the peptide in a "peptide binding site" or

groove which is structurally positioned at the end of the molecule farthest from the cell membrane. The peptide, or antigen, binding site, is also called the "antigen binding pocket" or "MHC groove." (Brown (1993) *Nature* 364:33-39; Stern (1994) *Structure* 2:245-251).

The binding of a peptide to a peptide binding site is dependent on the primary amino acid sequence of both the peptide and the Class II molecule. Only high affinity binding between a peptide and a Class II polypeptide will form a complex which will be presented extracellularly to T cells. The affinity, or strength, of this intermolecular attraction is determined by the same factors that exist for all peptide:polypeptide binding reactions, *e.g.*, conformation, hydrogen bonding, charge, ionic interactions. The limitations created by primary sequence are further limited by a size limitation; the peptide binding site is believed to be able to accomodate peptides ranging from about eight to about twenty amino acids in length (the "agretope" is that portion of the peptide recognized by the MHC molecule). As a result, different portions of an antigenic polypeptide are typically presented by different Class II molecules. Thus, when a peptide is internalized by an APC and proteolytically processed into fragments, only a single or small number of peptides will bind with high affinity, *i.e.*, be "specifically bound," to a specific Class II molecule. As only a limited number of Class II molecules are expressed in an individual, only a limited repetoire of peptides from a polypeptide antigen are presented.

For example, in the case of MS, a single region of MBP is involved in binding a limited set of Class II molecules to help generate anti-myelin autoreactive T cell clones. In some MS patients, a putative pathognomonic "immunodominant" MBP peptide and the Class II molecules to which it binds are suggested to be the MBP peptide spanning residues 83 to 102 (MBP 83-102) which binds to either the Class II DR allele designated DRB1*1501 and or to DRB5*0101 (other Class II alleles and MBP peptides may also be mediating MS or other demyelinating diseases in different individuals).

Recognition of the peptide:Class II complex by a helper CD4+ T lymphocyte is mediated by the binding of the T cell's clonally specific receptor (TCR) to the complex. The TCR's affinity to the complex is determined by its attraction to both the Class II molecule and the peptide occupying the antigen binding pocket (the portion of the peptide recognized by TCR is called the "epitope"). High affinity TCR binding to the APC complex activates the T cell, inducing its clonal proliferation and secretion of immunomodulatory cytokines (which

can be stimulatory or immunosuppressive). Thus, there is an amplification of an immune response specific for the antigenic peptide (the epitope) that is displayed by the Class II polypeptide. If the peptide is a self peptide, the response can be an autoimmune reaction.

Interfering with the ability of peptide:Class II complex binding to TCR can inhibit the development of or suppress an autoimmune reaction. For example, administration of antibodies to MHC Class II polypeptides can interfere with complex:TCR binding and the resultant pathogenic immune reaction. Antibodies to autoreactive TCR or T cell clones can have the same effect.

An alternative immunosuppressive strategy can be used to exploit the need for a co-stimulatory signal to the T cell by the APC. To activate CD4+ T cells, TCR binding by Class II:peptide is not sufficient. An additional, "co-stimulatory" signal is needed (not an MHC molecule). Typically, the required co-stimulatory signal is provided by an APC cell surface protein. Significantly, interaction of Class II:peptide with TCR without APC co-stimulation not only does not induce T cell activation, but it also results in a state of antigen-specific unresponsiveness on rechallenge, known as anergy *in vitro* and tolerance *in vivo* (Boussiotis (1994) *Curr. Opin. Immunol.* 6:797-807; Park (1997) *Eur. J. Immunol.* 27:1082-1090). This suppression and re-challenge non-responsiveness has been hypothesized to be by T cell clonal anergy or by a nonresponsiveness induced by immunosuppressive cytokines (Schwartz (1989) *Cell* 57:1073-1081; Quill (1987) *J. Immunol.* 138:3704-3712).

While the twenty amino acid long MBP 83-102 may be the immunodominant, naturally processed MBP peptide in some individuals, synthetic or recombinant modifications of myelin or peptide MBP 83-102 which bind to Class II molecule and/or T cell receptor with greater affinity can be therapeutically efficacious. Such peptides would be better reagents to inhibit the pathogenic autoimmune reaction, *e.g.*, through their administration as tolerance-inducing soluble Class II:peptide complexes. By providing novel MBP peptides capable of binding to Class II molecules and TCRs, the present invention fulfills these and other needs.

Current treatment for autoimmune disease consists primarily of treating the symptoms, but not intervening in the etiology of the disease. Broad spectrum immunosuppressive agents which have numerous undesirable side effects are typically employed. The inadequate treatments presently available illustrate the urgent need to identify new agents that prevent or suppress MHC-restricted immune responses, but avoid undesirable

side effects, such as nonspecific suppression of an individual's overall immune response. Compounds capable of selectively suppressing autoimmune responses at the helper CD4+ T cell level would provide a safer, more effective treatment. By providing novel MBP peptides and peptide:Class II molecule complexes, the present invention fulfills these and other needs.

SUMMARY OF THE INVENTION

The invention provides an isolated myelin basic protein (MBP) peptide, said peptide characterized by having an amino acid sequence Phe -X - Lys - Asn - Ile - Val - X - X - X - Thr - X -X, wherein X is any amino acid. In one embodiment, the isolated myelin basic protein peptide is characterized by having an amino acid sequence Phe - Phe - Lys - Asn - Ile - Val - Thr - Pro - Arg - Thr - Pro - Pro, or a conservative substitution thereof. The isolated myelin basic protein peptide can be linked to a heterologous sequence. In one embodiment, linkage of the myelin basic protein peptide to a heterologous sequence results in a fusion protein.

The invention also provides an isolated myelin basic protein peptide which specifically binds to an antibody directed against a peptide characterized by having an amino acid sequence Phe - Phe - Lys - Asn - Ile - Val - Thr - Pro - Arg - Thr - Pro - Pro.

In another embodiment, the invention provides an isolated nucleic acid encoding a myelin basic protein peptide, said peptide characterized by having an amino acid sequence Phe - X - Lys - Asn - Ile - Val - X - X - X - Thr - X - X , wherein X is any amino acid. The encoded myelin basic protein peptide can be characterized by having an amino acid sequence Phe - Phe - Lys - Asn - Ile - Val - Thr - Pro - Arg - Thr - Pro - Pro , or a conservative substitution thereof. In one embodiment, the isolated nucleic acid comprises SEQ ID NO:1.

The invention also provides a composition comprising an MHC Class II complex capable of binding a T cell receptor, the complex consisting essentially of: an MHC Class II polypeptide comprising an extracellular domain of an MHC Class II molecule sufficient to form an antigen binding pocket, said component being encoded by an allele associated with an autoimmune disease directed to myelin basic protein, wherein the Class II component is soluble under physiological conditions in the absence of detergent or lipid; and, a myelin basic protein having an amino acid sequence Phe -X - Lys - R_1 - Ile - Val - X -

X - X - Thr - X -X , wherein X is any amino acid, and R_1 is Asn or Gln; wherein the myelin basic protein peptide is bound to the MHC Class II component antigen binding pocket. The myelin basic protein peptide can have an amino acid sequence Phe - Phe - Lys - Asn - Ile - Val - Thr - Pro - Arg - Thr - Pro - Pro, or a conservative substitution thereof. The composition can comprise a fusion protein and/or an effector composition. In alternative embodiments, the autoimmune disease is multiple sclerosis, or the class II polypeptide comprises the antigen binding pocket of an HLA DR2.

The invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a pharmacologically effective amount of a peptide characterized by having an amino acid sequence Phe -X - Lys - Asn - Ile - Val - X - X - X - Thr - X -X, wherein X is any amino acid.

The invention also provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a pharmacologically effective amount of a composition comprising an MHC class II complex capable of binding a T cell receptor, the complex consisting essentially of: an MHC class II polypeptide comprising an extracellular domain of an MHC class II molecule sufficient to form an antigen binding pocket, said component being encoded by an allele associated with an autoimmune disease directed to myelin basic protein, wherein the class II component is soluble under physiological conditions in the absence of detergent or lipid; and, a myelin basic protein having an amino acid sequence Phe -X - Lys - R_1 - Ile - Val - X - X - X - Thr - X -X , wherein X is any amino acid, and R_1 is Asn or Gln; wherein the myelin basic protein peptide is bound to the MHC class II component antigen binding pocket.

The invention provides an antibody, specifically immunoreactive under immunologically reactive conditions, to a myelin basic protein peptide, said peptide characterized by having an amino acid sequence Phe - Phe - Lys - Asn - Ile - Val - Thr - Pro - Arg - Thr - Pro - Pro. In another embodiment, the invention provides an antibody is specifically immunoreactive under immunologically reactive conditions, to a myelin basic protein peptide comprising the peptide encoded by a nucleic acid of encoding a myelin basic protein peptide, said peptide characterized by having an amino acid sequence Phe - X - Lys - Asn - Ile - Val - X - X - X - Thr - X - X , wherein X is any amino acid.

The invention provides a method for inhibiting a T cell mediated immune response against myelin basic protein in a subject, comprising administering to the subject an isolated myelin basic protein (MBP) peptide, said peptide characterized by having an amino acid sequence Phe -X - Lys - Asn - Ile - Val - X - X - X - Thr - X -X, wherein X is any amino acid, in an amount effective to treat the T cell mediated immune response.

The invention provides a method for inhibiting a T cell mediated immune response against myelin basic protein in a subject, comprising administering to the subject a composition comprising an MHC Class II complex capable of binding a T cell receptor, the complex consisting essentially of: an MHC Class II polypeptide comprising an extracellular domain of an MHC Class II molecule sufficient to form an antigen binding pocket, said component being encoded by an allele associated with an autoimmune disease directed to myelin basic protein, wherein the Class II component is soluble under physiological conditions in the absence of detergent or lipid; and, a myelin basic protein having an amino acid sequence Phe -X - Lys - R_1 - Ile - Val - X - X - X - Thr - X -X , wherein X is any amino acid, and R_1 is Asn or Gln; wherein the myelin basic protein peptide is bound to the MHC Class II component antigen binding pocket.

The invention provides a method for inhibiting a T cell mediated immune response against myelin basic protein in a subject wherein the T cell mediated immune response causes a pathology to a neurological system. In one embodiment, the pathology to the neurological system is characterized as multiple sclerosis.

The invention provides a method of identifying a T cell epitope on an antigen which, when bound to the antigen binding pocket of an MHC Class II molecule, is capable of binding to a T cell receptor, such binding triggering an extracellular acidification reaction by a T cell expressing the T cell receptor, said method comprising the steps of: a) providing a composition comprising the T cell epitope bound to the antigen binding pocket of an MHC Class II molecule; b) contacting a T cell expressing the T cell receptor with the epitope; and, c) measuring the extracellular acidification, wherein a change in the extracellular acidification indicates the binding of T cell epitope to the T cell receptor. In one embodiment, the change in extracellular acidification is measured using a microphysiometer.

A further understanding of the nature and advantages of the present invention may be realized by reference to the remaining portions of the specification, the figures and claims.

All publications, patents and patent applications cited herein are hereby expressly incorporated by reference for all purposes.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A shows the MBP peptides used in the studies described in Example 1. Figure 1B shows MBP peptides with various alanine substituted residues used in the studies described in Example 1.

Figures 2A, 2B and 2C show the results of analyses described in Example 1, demonstrating increased levels of gamma-IFN, TNF-beta and extracellular acidification rates, respectively, with six N-terminal truncated MBP peptides. Figures 2D, 2E and 2F show results of analyses described in Example 1, comparing the gamma-IFN, TNF-beta and acidification rates, respectively, of five N-terminus truncated MBP peptides.

Figures 3A, 3B, and 3C, show the results of analyses described in Example 1, comparing the gamma-IFN, TNF-beta and acidification rates, respectively, induced by C-terminal truncated MBP peptides. Figures 3D, 3E, and 3F, show the results of analyses described in Example 1, comparing the gamma-IFN, TNF-beta and acidification rates, respectively, induced by C-terminal truncated MBP peptides.

Figures 4A, 4B and 4C show the results of analyses described in Example 1, demonstrating MBP peptide residues necessary to induce gamma-IFN, TNF-beta along with increased acidification rates, respectively, when presented to SS8T cells by Class II DRB5*0101. Figures 4D, 4E and 4F show the results of analyses described in Example 1, demonstrating MBP peptide residues necessary to induce gamma-IFN, TNF-beta along with increased acidification rates, respectively, when presented to SS8T cells by Class II DRB5*0101.

Figure 5A shows, in highlights (boxed area), the core TCR recognition sequence as MBP(91-100) (SEQ ID NO:38). Figure 5B shows (by arrows) the important MBP amino acid residues involved in TCR contact as F-91, K-93, N-94, I-95, and V-96.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to methods and compositions that can be used to inhibit those aspects of the immune system which are responsible for undesirable autoimmunity. This invention is a first description of a novel set of peptides derived from human myelin basic protein (MBP) that can be used in the treatment of autoimmune mediated demyelinating disease, in particular, multiple sclerosis (MS). The invention provides therapeutic compositions comprising novel MBP peptides for, *e.g.*, inducing oral or general tolerance. Also provided are a soluble MBP peptide:Class II molecule complex for targeting T cells mediating the autoimmune reaction. The complex targets T cell clones reactive to MBP by binding to autoreactive T cell receptor (TCR) capable of binding the MBP:Class II complex. The binding of the MBP:Class II complex to the T cell is therapeutically efficacious for inhibiting the development of or treating autoimmune demyelinating disease. While the invention is not limited by any particular mechanism of action, the compositions of the invention are pharmaceutically active by, *e.g.*, inducing T cell clonal anergy and tolerance or inducing a cytokine-mediated immunosuppressive immune response.

The invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a pharmacologically effective amount of a peptide characterized by having an amino acid sequence Phe -X - Lys - Asn - Ile - Val - X - X - X - Thr - X -X, wherein X is any amino acid. Administration of the peptide alone can be used to induce oral tolerance, which is a phenomenon of systemic, antigen specific, immunological hyporesponsiveness that results from oral administration of a protein. The mechanism by which oral tolerance is generated depends on the amount of antigen (peptide) administered. Low doses favor induction of regulatory immunosuppressive helper CD4+ T cells. Higher doses favor clonal deletion and anergy (tolerance). Regulatory T cells induced by low doses of oral antigen, when re-stimulated, secrete immunosuppressive cytokines that suppress the inflammatory response in an antigen nonspecific manner. Orally administered autoantigens have been shown to suppress a wide variety of experimental autoimmune diseases, including the most widely studied animal model for MS, the murine demyelinating disease experimental autoimmune encephalomyelitis (EAE). Murine MBP peptide has been used to generate an antigen-specific oral tolerance and to prevent development of acute EAE (Bitar (1988) *Cell. Immun.* 112:364-370; Higgins (1988) *J. of Immun.* 140:440-445; Khoury (1992) *J. Exp. Med.*

176:1355-1364; Whitacre (1996) *Clin. Immunol. Immunopathol.* 80:S31-S39). In one study, oral administration of bovine myelin to MS patients induced T cells that secreted the immunosuppressive cytokine TGF-beta-1 (Fukaura (1996) *J. Clin. Invest.* 98:70-77). Thus, the novel human MBP peptides of the invention can be efficacious in the generation of an antigen-specific immunosuppressive reaction, as in the induction of oral tolerance.

The present invention is also directed to purified complexes comprising at least an effective antigen-binding portion of a dimeric Class II molecule or a single chain (monomeric) subunit of a Class II and an MBP peptide of the invention. An "effective antigen-binding portion" is the mininum amount of dimeric or monomeric Class II molecule necessary to bind MBP peptide with sufficient affinity to create a complex capable of being recognized by and specifically bind to an autoreactive TCR. The complex of the invention, comprising MBP peptide and the appropriate Class II molecule, can be bound or otherwise associated together covalently or noncovalently. A third component, such as an "effector" component, can also be included in the complex of the invention. The effector component can be a cytotoxic agent, *i.e.*, a toxin, radioisotope, apoptotic-inducing agent, and the like, to selectively eliminate or neutralize the targeted, autoimmune T cell population.

In other aspects, the invention is directed to pharmaceutical compositions wherein the peptide and complexes of the invention are the active ingredients. These compositions are used to down-regulate or eliminate autoreactive components of the immune system and to treat autoreactive, demyelinating, T cell mediated immune responses. The invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a pharmacologically effective amount of the MBP peptide:Class II complex. The complexes selectively bind MBP autoreactive T cells. While the invention is not limited to any specific means by which the compositions can be pharmaceutically active, the peptides and complexes can induce clonal anergy/ tolerance or induce a cytokine-mediated immunosuppressive immune response. The complexes can be further conjugated to cytotoxic agents to specifically eliminate targeted autoreactive T cells.

I. Nucleic Acids Encoding MBP Peptides and MHC Class II DR Alleles and Recombinant Expression of these Nucleic Acids

This invention provides novel myelin basic protein (MBP) peptides and complexes of these MBP peptides and MHC Class II polypeptides comprising at least the extracellular domain of a Class II molecule sufficient to form an antigen binding pocket, and nucleic acid encoding these MBP peptides and Class II polypeptides. In addition to providing synthetic forms of MBP and Class II nucleic acids, the invention provides recombinantly generated nucleic acid, MBP peptides, and Class II polypeptides. As nucleic acid encoding these peptides and proteins can be expressed *in vitro* or *in vivo*, the invention provides for a variety of means of expressing these sequences, including expression cassettes, vectors, cell lines, transgenic plants and animals, and the like.

The invention can be practiced in conjunction with any method or protocol known in the art, which are well described in the scientific and patent literature. Therefore, only a few general techniques will be described prior to discussing exemplary methodologies and examples relative to the novel reagents and methods of the invention.

General Techniques

The MBP peptide- and MBP:Class II complex-encoding genes and nucleic acids of this invention, whether RNA, cDNA, genomic DNA, or hybrids thereof, can be isolated from a variety of sources; genetically engineered; expressed recombinantly; or synthesized *in vitro*. Nucleic acids encoding for the MBP and MBP:Class II complexes of the invention can be expressed in transgenic plants and animals, transformed cells and cell lines, in a transformed cell lysate, or in a partially purified or a substantially pure form. Techniques for nucleic acid manipulation of genes encoding the MBP and MBP:Class II complexes of the invention, such as site specific mutagenesis, generating libraries, subcloning into expression vectors, labeling probes, sequencing DNA, DNA hybridization are described in the scientific and patent literature, see *e.g.*, Sambrook, ed., MOLECULAR CLONING: A LABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989) ("Sambrook"); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel, ed. John Wiley & Sons, Inc., New York (1997) ("Ausubel"); and, LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY: HYBRIDIZATION WITH NUCLEIC ACID PROBES, *Part I. Theory and Nucleic Acid Preparation*, Tijssen, ed. Elsevier, N.Y. (1993) ("Tijssen"). Sequencing methods typically

use dideoxy sequencing (Sequenase, U.S. Biochemical), however, other kits and methods are available and well known to those of skill in the art.

Nucleic acids and proteins are detected, isolated and quantified in accordance with the teachings and methods of the invention described herein by any of a number of general means well known to those of skill in the art. These include, *e.g.*, analytical biochemical methods such as NMR, spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and hyperdiffusion chromatography, various immunological methods, such as fluid or gel precipitin reactions, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immuno-fluorescent assays, Southern analysis, Northern analysis, Dot-blot analysis, gel electrophoresis, RT-PCR, quantitative PCR, other nucleic acid or target or signal amplification methods, radiolabeling, scintillation counting, and affinity chromatography.

Mutations can be introduced into a nucleic acid by a variety of conventional techniques, well described in the scientific and patent literature. For example, one rapid method to perform site-directed mutagenesis efficiently is the overlap extension polymerase chain reaction (OE-PCR) (Urban (1997) *Nucleic Acids Res.* 25:2227-2228).

MHC Class II DR Alleles

The invention provides nucleic acids encoding novel myelin basic protein (MBP) peptides and MHC Class II polypeptides comprising at least the extracellular domain of an MHC Class II molecule sufficient to form an antigen binding pocket. The invention provides an example of human DR Class II alleles encoding polypeptide capable of binding the MBP peptides of the invention, specifically, the alleles designated DRB1*1501 and DRB5*0101 (see, e.g., Weber (1993) *Proc. Natl. Acad. Sci. 90:11049-11053*). However, the complexes of the invention are not limited to these DR alleles or their corresponding Class II polypeptides. The MBP:Class II complexes of the invention include any polypeptide comprising an MHC Class II antigen binding pocket, or functional equivalent thereof, which can bind an MBP peptide of the invention with sufficient affinity to be used in the methods of the invention. Means to identify known such DR alleles and Class II polypeptides are well known in the scientific and patent literature, *e.g.*, through electronic databanks, as Medline, GenBank, and the like. Means to identify MHC Class II DR alleles encoding polypeptide

having an antigen binding pocket capable of binding a particular peptide, such as, *e.g.*, an MBP peptide of the invention, are also well known in the scientific and patent literature (see, *e.g.*, Rammensee (1995) *Immunogenetics* 41:178-228; Sinigaglia (1994) *Curr. Opin. Immunol.* 6:52-56).

In one embodiment of the invention, the Class II:peptide complexes are water soluble. Water solubility can be engineered in Class II polypeptides by deleting transmembrane domain (typically hydrophobic) amino acid residues. This is most effectively accomplished by recombinantly redesigning the DR allele and expressing the truncated Class II molecule. Deletion of the transmembrane domain can be by outright deletion of residues or by recombinantly redesigning Class II coding sequence to substitute hydrophobic residues with hydrophilic residues. This also facilitates recovery of the water-soluble Class II polypeptide after recombinant expression. For example, the transmembrane-inactivated Class II can be secreted directly into the culture medium of recombinant expression hosts. Further deletion of cytoplasmic or extracellular residues can be effective in eliminating potentially immunogenic epitopes. In one embodiment, a nucleic acid is designed to recombinantly express a minimal peptide binding site/TCR binding polypeptide, *i.e.*, only the amount of polypeptide needed to bind the MBP peptide of the invention with sufficient affinity to be recognized by the appropriate autoreactive TCR and to be used in the methods of the invention. The peptide-binding polypeptide, whether in the form of a minimal peptide binding site molecule, an isolated or recombinant full length, alterred or truncated Class II molecule, or the like, can be in dimeric or moneric form.

Additional Class II polymorphic DR alleles can be identified and characterized using various methods, including: i) computer searches of DNA databases for DNAs containing sequences conserved with the Class II DR molecules described above, ii) hybridization with a probe from a known DR gene sequence to mRNA, cDNA or DNA sequence or libraries, and, iii) by PCR or other signal or target amplification technologies using primers complementary to regions highly conserved among different DR genes.

Nucleic acid amplification methods can also used to identify, isolate and generate Class II polymorphic alleles. Suitable amplification methods include, but are not limited to: polymerase chain reaction, PCR (PCR PROTOCOLS, A GUIDE TO METHODS AND APPLICATIONS, *ed.* Innis, Academic Press, N.Y. (1990) and PCR STRATEGIES (1995), *ed.*

Innis, Academic Press, Inc., N.Y. (Innis)); ligase chain reaction (LCR) (Wu (1989) *Genomics* 4:560; Landegren (1988) *Science* 241:1077; Barringer (1990) *Gene* 89:117); transcription amplification (Kwoh (1989) *Proc. Natl. Acad. Sci. USA* 86:1173); and, self-sustained sequence replication (Guatelli (1990) *Proc. Natl. Acad. Sci. USA,* 87:1874); Q Beta replicase amplification (Smith (1997) *J. Clin. Microbiol.* 35:1477-1491, automated Q-beta replicase amplification assay; Burg (1996) *Mol. Cell. Probes* 10:257-271) and other RNA polymerase mediated techniques (*e.g.*, NASBA, Cangene, Mississauga, Ontario); see also Berger (1987) *Methods Enzymol.* 152:307-316, Sambrook, Tijssen, and Ausubel, as well as Mullis (1987) U.S. Patent Nos. 4,683,195 and 4,683,202.

Sequencing of Nucleic Acid

Sequencing of isolated MBP- and Class II- encoding nucleic acid is used, *e.g.*, to identify and characterize allelic DR species encoding polypeptides capable of binding MBP peptides; to confirm sequences of synthetically manufactured or cloned nucleic acid; confirm mutations; and the like. MBP- and Class II-encoding sequences can be sequenced as inserts in vectors, as inserts released and isolated from the vectors or in any of a variety of other forms (*i.e.*, as amplification products). MBP- and Class II-encoding inserts can be released from the vectors by restriction enzymes or amplified by PCR or transcribed by a polymerase. For sequencing of the inserts to identify full length coding sequences, primers based on the N- or C- terminus, or based on insertion points in the original phage or other vector, can be used. A variety of nucleic acid sequencing techniques are well known and described in the scientific and patent literature, see, *e.g.* Rosenthal (1987) *supra*; Arlinghaus (1997) *Anal. Chem.* 69:3747-3753; Dubiley (1997) *Nucleic Acids Res.* 25:2259-2265; for use of biosensor chips for identification and sequencing of nucleic acids.

Expression of Recombinant MBP Peptides and Class II Polypeptides

The invention provides methods and reagents for recombinant expression of the novel MBP peptides and Class II molecules used in the complexes of the invention. The nucleic acids of the invention may be introduced into a genome or into the cytoplasm or a nucleus of a cell and expressed by a variety of conventional techniques, well described in the scientific and patent literature. See, *e.g.*, Roberts (1987) *Nature* 328:731; Berger (1987) *supra*; Schneider (1995) *Protein Expr. Purif.* 6435:10; Sambrook, Tijssen or Ausubel. Product information from manufacturers of biological reagents and experimental equipment

also provide information regarding known biological methods. The promoters and vectors used in this invention can be isolated from natural sources, obtained from such sources as ATCC or GenBank libraries, or prepared by synthetic or recombinant methods, as described herein. A few selected illustrative general and specific teaching examples relevant to such technology are described below.

Vectors and Transcriptional Control Elements

The invention, providing methods and reagents for making the novel nucleic acids described herein, further provides methods and reagents for expressing these nucleic acids using novel expression cassettes, vectors, transgenic plants and animals, using constitutive and inducible transcriptional and translational *cis*- (*e.g.*, promoters and enhancers) and *trans*-acting control elements. The expression of natural, recombinant or synthetic MBP peptide- or polypeptide-encoding nucleic acids can be achieved by operably linking the coding region a promoter (that can be tissue-specific, constitutive or inducible), incorporating the construct into an expression cassette (such as an expression vector), and introducing the resultant construct into an *in vitro* reaction system or a suitable host cell or organism. Synthetic procedures may also be used to generate any nucleic acid of the invention. Transcriptional and translational control elements include transcription and translation initiation sequences, promoters and enhancers, transcription and translation terminators, polyadenylation sequences, and other sequences useful for transcribing DNA into RNA. In construction of recombinant expression cassettes, vectors, transgenics, of the invention, a promoter fragment can be employed to direct expression of the desired nucleic acid in all tissues of a plant or animal. Promoters that drive expression continuously under physiological conditions are referred to as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. The expression systems optionally at least one independent terminator sequence, sequences permitting replication of the cassette *in vivo, e.g.*, plants, eukaryotes, or prokaryotes, or a combination thereof, (*e.g.*, shuttle vectors) and selection markers for the selected expression system, *e.g.*, plant, prokaryotic or eukaryotic systems. To ensure proper polypeptide expression under varying conditions, a polyadenylation region at the 3'-end of the coding region can be included.

The nucleic acids of the invention can be expressed in expression cassettes, vectors or viruses which are transiently expressed in cells using, *e.g.*, episomal expression

systems. Expression vectors capable of expressing proteins are well known in the art. Selection markers can be incorporated into expression cassettes and vectors to confer a selectable phenotype on transformed cells and sequences coding for episomal maintenance and replication such that integration into the host genome is not required. For example, the marker may encode antibiotic resistance, particularly resistance to chloramphenicol, kanamycin, G418, bleomycin, hygromycin, or herbicide resistance, such as resistance to chlorosulfuron or Basta, to permit selection of those cells transformed with the desired DNA sequences, see for example, Blondelet-Rouault (1997) *Gene* 190:315-317; Aubrecht (1997) *J. Pharmacol. Exp. Ther.* 281:992-997 . Because selectable marker genes conferring resistance to substrates like neomycin or hygromycin can only be utilized in tissue culture, chemoresistance genes are also used as selectable markers *in vitro* and *in vivo*.

Production of Transformants and Transgenic Plants and Animals

The invention provides for a variety of *in vivo* systems expressing the MBP peptides, Class II polypeptides, and peptide:Class II complexes of the invention, including transformed cells and transgenic plants and animals. *In vivo* expression systems that can be used include bacterial, yeast, insect (baculovirus), plant, and mammalian cell systems. The system used will depend on a variety of factors, including activities and amounts desired.

There are several well-known methods of introducing nucleic acids into animal, plant, bacterial and other cells, a process often called "transforming," any of which may be used in the methods of the present invention (see, *e.g.*, Sambrook, Ausubel or Tijssen). *In vivo* expression systems and techniques for transforming a wide variety of animal and plant cells are well known and described in the technical and scientific literature. See, *e.g.*, Weising, *Ann. Rev. Genet.* 22:421-477 (1988) for plant cells and Sambrook or Tijssen for animal and bacterial cells.

II. MBP Peptides and MHC Class II DR Polypeptides

This invention provides novel myelin basic protein (MBP) peptides, Class II polypeptides comprising at least an amount of polypeptide needed to bind an MBP peptide of the invention with sufficient affinity to be recognized by the appropriate autoreactive TCR, and Class II:MBP peptide complexes. The invention provides isolated (from natural sources), synthetic, and recombinantly generated forms of MBP peptides and Class II polypeptides.

These peptides and proteins can be recombinantly expressed *in vitro* or *in vivo*. The peptides, polypeptides and complexes of the invention can be made and isolated using any method known in the art, and the invention provides a few exemplary means for generating such proteins. In addition, means to make Class II:peptide complexes are taught in, *e.g.*, U.S. Patent Nos. (USPN) 5,194,425, issued March 16, 1993; 5,130,297, issued July 14, 1992; 5,284,935, issued February 8, 1994; 5,260,422, issued November 9, 1993; and 5,468,481, issued November 21, 1995.

MBP peptides and Class II:peptide complexes of the invention can be synthesized, whole or in part, using chemical methods well known in the art (see *e.g.*, Caruthers (1980) *Nucleic Acids Res. Symp. Ser.* 215-223; Horn (1980) *Nucleic Acids Res. Symp. Ser.* 225-232; Banga, A.K., *Therapeutic Peptides and Proteins, Formulation, Processing and Delivery Systems* (1995) Technomic Publishing Co., Lancaster, PA ("Banga")). For example, peptide synthesis can be performed using various solid-phase techniques (see *e.g.*, Roberge (1995) *Science* 269:202; Merrifield (1997) *Methods Enzymol.* 289:3-13) and automated synthesis may be achieved, *e.g.*, using the ABI 431A Peptide Synthesizer (Perkin Elmer) in accordance with the instructions provided by the manufacturer.

The newly synthesized peptide can be isolated and substantially purified by preparative high performance liquid chromatography (HPLC), see *e.g.*, Creighton, *Proteins, Structures and Molecular Principles*, WH Freeman and Co, New York NY, 1983. The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (*e.g.*, the Edman degradation procedure; Creighton, *supra*). Laser desorption mass spectrometry (MALDI-MS) can also be used to evaluate the progress of peptide synthesis at all the necessary levels, including automated assembly, cleavage and deprotection chemistries, RP-HPLC analyses and purifications, and structural validation of the final product (Moore (1997) *Methods Enzymol.* 289:520-542). Electrospray ionization mass spectrometry is an easy, rapid method for the verification of proper peptide synthesis and for the identification of most synthetic by-products (Burdick (1997) *Methods Enzymol.* 289:499-519).

Additionally the amino acid sequences of the peptides and polypeptides of the invention, or any part thereof, can be alterred during direct synthesis and/or combined using chemical methods with sequences from other proteins, or any part thereof, to produce a variant polypeptide. Modified peptides and proteins of the invention can be produced by, in addition

to manipulation of nucleic acid coding sequence, *e.g.*, with site-directed mutagenesis, chemical modification of polypeptide to introduce unnatural amino acid side chains (see *e.g.*, Paetzel (1997) *J. Biol. Chem.* 272:9994-10003, for general methodology). As another example, for site-specific incorporation of biotin-containing amino acid biocytin, see Gallivan (1997) *Chem. Biol.* 4:739-749; for site-specific incorporation of unnatural amino acids into proteins *in vivo*, see *e.g.*, Liu (1997) *Proc. Natl. Acad. Sci. USA* 94:10092-10097; see also Koh (1997) *Biochemistry* 36:11314-11322.

Class II polypeptides suitable for use in the present invention can also be isolated from a natural sources, such as a cell line expressing the appropriate DR allele or a patient of the appropriate genotype, using a variety of techniques well known in the art. For example, the cells can be solubilized by treatment with papain, by treatment with 3M KCl, or by treatment with detergent. Detergent extraction of Class II protein from lymphocytes followed by affinity purification can also be used. Detergent can then be removed by dialysis or selective binding beads, *e.g.*, Bio Beads. The molecules can be obtained by isolation from any cell expressing the Class II molecule of interest, such as, *e.g.*, B or T lymphocytes from an individual with the appropriate genotype, as one suffering from demyelinating autoimmune disease. Suitable MHC molecules can be isolated from B or T cells which have been immortalized by transformation, *e.g.*, as by B cell transformation with a replication deficient Epstein-Barr virus, utilizing techniques known in the art.

Isolation of individual subunits from the isolated Class II molecule is easily achieved using standard techniques known to those skilled in the art. For example, alpha and beta subunits from Class II molecules can be separated using SDS/PAGE and electroelution. (see *e.g.*, Rothenhausler (1990) *Proc. Natl. Acad. Sci. USA* 87:352-354; Gorga (1987) *J. Biol. Chem.* 262:16087-16094; Dornmair (1989) *Cold Spring Harbor Symp. Quant. Biol.* 54:409-416). One of skill will recognize that a number of other standard methods of separating molecules can be used, such as, *e.g.*, ion exchange chromatography, size exclusion chromatography, gel permeation chromatography, HPLC, RP-HPLC, or affinity chromatography. See, *e.g.*, Banga.

In one embodiment, an additional "effector" composition is linked to the complexes of the invention to inhibit or abrogate the autoimmune reaction. The "effector" portion of the molecule can be, *e.g.*, a toxin, a chemotherapeutic agent, an antibody to a

cytotoxic T lymphocyte (CTL) surface molecule, a lipase, or a toxic radioisotope emitting, *e.g.*, gamma radiation from radioisotopes such as yttrium-90, phosphorus-32, lead-212, iodine-131, or palladium-109. A number of protein toxins are well known in the art including, *e.g.*, ricin, diphtheria, gelonin, *Pseudomonas* toxin, and abrin. Chemotherapeutic agents include, *e.g.*, doxorubicin, daunorubicin, methotrexate, cytotoxin, and anti-sense RNA. Antibiotics can also be used. In some cases the toxin or other effector component is entrapped in a delivery system such as a liposome or dextran carrier; in these cases, either the active component or the carrier may be bound to the Class II:peptide complex.

In preparing pharmaceutical compositions of the present invention, it is frequently desirable to modify the peptides or complexes to alter their pharmacokinetics and biodistribution (discused further below). For instance, methods suitable for increasing serum half-life of the complexes include treatment to remove carbohydrates which are involved in the elimination of the complexes from the bloodstream. Preferably, substantially all of the carbohydrate moieties are removed by the treatment. Substantially all of the carbohydrate moieties are removed if at least about 75%, preferably about 90%, and most preferably about 99% of the carbohydrate moieties are removed. Conjugation to soluble macromolecules, such as proteins, polysaccharides, or synthetic polymers, such as polyethylene glycol, is also effective. Other methods include protection of the complexes in vesicles composed of substances such as proteins, lipids (for example, liposomes, discussed below), carbohydrates, or synthetic polymers.

Formation of the Complex

The Class II polypeptide:MBP peptide complex can be formed by any standard means known in the art. For example, the peptides of the invention can be associated noncovalently with antigen binding sites by, *e.g.*, simply mixing the two components. They can also be covalently bound to the antigen binding pocket using standard procedures by, *e.g.*, photoaffinity labelling (see, *e.g.*, Hall (1985) *Biochemistry* 24:5702-5711; Leuscher (1990) *J. Biol. Chem.* 265:11177-11184; Wraith (1989) Cell 59:247-255). Other modes of linkage include, *e.g.*, attachment via carbohydrate/lectin groups on the glycoproteins (Husain (1995) *Biochem. Mol. Biol. Int.* 36:669-677), as the carbohydrate moieties of Class II alpha and/or beta chains. Dehydration reactions using carbodiimides can be used. Heterobifunctional linkers such as N-hydroxysuccinimide ester (NHS), N-succinimidyl 3-(2-pyridyldithio)-

propionate (SPDP), substituted 2-iminothiolanes, glutaraldehyde and the like can also be used (see, *e.g.*, Traut (1995) *Biochem. Cell Biol.* 73:949-958; Haselgrubler (1995) *Bioconjug Chem.* 6:242-248; Carroll (1994) *Bioconjug. Chem.* 5:248-256). Alternatively, the Class II: peptide complex can be designed as one contiguous recombinant polypeptide. See also, PCT Publication Nos. WO 96/40944, December 19, 1996; WO 96/40194, December 19, 1996; and, WO 97/04360, November 6, 1997.

Effectors or other fusion protein components (for purification, etc., discussed above) can also be attached to the complex using these methods. The sequence of preparing the complex depends on the components in each instance. For example, in an exemplary protocol, the peptide portion and MHC subunit component are noncovalently associated by contacting the peptide with the MHC subunit component, *e.g.*, by mixing. The effector is then covalently linked. Alternatively, the effector and MHC subunit may be first conjugated and this conjugate complexed with the MBP peptide component. If the effector is itself a protein, the entire complex may be made directly from the appropriate encoding DNA using recombinant methods.

Assessment of the Complex

The peptides and MBP:Class II complexes of the invention can be assayed using a variety of *in vitro* models well known in the art. As discussed above, to activate CD4+ T cells, TCR binding by Class II:peptide is not sufficient. An additional, "co-stimulatory" signal is needed. Interaction of a Class II:peptide complex of the invention with TCR lacks a co-stimulatory signal. Thus, a state of antigen-specific T cell non-responsiveness is induced (Boussiotis (1994) *Curr. Opin. Immunol.* 6:797-807; Park (1997) *Eur. J. Immunol.* 27:1082-1090). This "tolerance" or "anergy" immunosuppression and re-challenge non-responsiveness can be caused by T cell clonal anergy, by a nonresponsiveness induced by immunosuppressive cytokines, or both (Schwartz (1989) *Cell* 57:1073-1081; Quill (1987) *J. Immunol.* 138:3704-3712).

In one exemplary *in vitro* system, the complex is incubated with autoreactive T cells and the cells are rechallenged with MBP. Peripheral blood T cells from patients with a demyelinating disease, such as MS, or myelin- (MBP-) reactive T cell clones can be used. T cells can be isolated before the analysis. The autoreactive T cells can be restimulated with antigen (MBP or myelin) *in vitro* (using syngeneic APCs) before administration of the

putative toleragen. "Resting" T cells (cells not stimulated *in vitro*) can be used. The T cells are treated with varying amounts of a complex of the invention for varying amounts of time. The binding of the complexes to the MBP-reactive T cells results in inhibiting or abrogating the *in vitro* autoimmune reaction.

The degree of immunosuppression or re-challenge non-responsiveness (i.e., tolerance, anergy), can be measured by monitering cell proliferation, cell metabolism, secretion of cytokines or lymphokines, or any form of cell activation. T cell activation can be measured by a variety of means well known in the art. For example, T cell proliferation can be assessed, *e.g.*, as measured by ^{3}H-thymidine uptake or by uptake of 3-(4,5-dimethyl-thiazol-2-7')-2,5 diphenyltetrazolium bromide (MTT) (see, *e.g.*, Liu (1997) *J. Neurochem.* 69:581-593). Alternatively, as T cells synthesize and secrete cytokines upon activation, the immunosuppressive efficacy of a Class II:peptide complex can be assessed by measuring cytokine transcription, translation or secretion. Thus, a variety of cytokines and lymphokines can be quantitated, *e.g.*, interleukins, interferons (INFs) (*e.g.*, gamma INF), tumor necrosis factors (TNFs) (*e.g.*, TNF beta) and the like. Cell death can also be monitered, as it has been observed that prolonged incubation of resting T cells with soluble MHC-Class II peptide complexes results in T cell apoptosis (Arimilli (1996) *Immunol. and Cell Biol.* 74:96-104). Cell death can be measured by a variety of known procedures, *e.g.*, dye exclusion permeability. Apoptosis can be assessed using, *e.g.*, cellular DNA fragmentation, observation (as with transmission electron microscopy), detection and quantitation of apoptosis-associating protein, as bcl-2, and the like (see, *e.g.*, Arimilli (1996) *supra*).

A preferred method of assessing the immunosuppressive efficacy of the Class II:peptide complexes of the invention is through the use of a microphysiometer to measure the rate of production of acidic metabolites in T cells. By using this apparatus, effect of the interaction of the complexes with TCRs can be quickly and easily detected. The earliest measurable event in the activation of a T cell after stimulation by a TCR:Class II:peptide complex binding reaction is an increase in the lymphocyte's metabolism, as reflected by its acidic byproducts. The microphysiometer measures the acidity of the principal catabolic products in mammalian cells, lactate and carbon dioxide. Very small changes in the acidity of the cultural medium bathing a small sample of cells can be readily determined with a light-addressable potentiometric sensor. The rate of acidification is used as a measure of catabolic

rate of the cells being assayed. See, *e.g.*, Parce (1989) *Science* 246:243-247; Owicki (1990) *Proc. Natl. Acad. Sci. USA* 87:4007-4011; Renschler (1995) *Cancer Res.* 55:5642-5647; Beeson (1996) *J. Exp. Med.* 184:777-782.

Addition of MBP peptide or myelin polypeptide to a mixture of autoreactive T cells and syngeneic APCs results in an increase in acid release due to the antigen specific binding of autoreactive TCR and APC-bound Class II:MBP peptide complexes. The immunosuppressive capability of an MBP peptide or Class II:MBP peptide complex of the invention can thus be evaluated by first adding a complex to an autoreactive T cell/APC culture followed by antigen (MBP or myelin) challenge. Lack of or a relative decrease in cell activation indicative of immunosuppression can be measured by a lessening or lack of extracellular acidification.

In one embodiment, a complex of the invention with an effector component is used. The treatment can be two-fold: the individual is first treated with the Class II:MBP peptide complex to down-regulate the immune system; further down-regulation is achieved by treatment with complex and an effector component.

III. Detection and Purification of Polypeptides

The invention also provides methods and reagents for isolating, detecting or quantitating the MBP peptides and Class II polypeptide complexes of the invention by a variety of methods.

Antibodies

In one embodiment, the invention provides antibodies specifically immunoreactive under immunologically reactive conditions to an MBP peptide of the invention. These Abs can be used in the isolation, detection or quantitation of the MBP peptides or complexes of the invention. Methods of producing polyclonal and monoclonal antibodies are known to those of skill in the art and described in the scientific and patent literature, see, *e.g.*, Coligan, CURRENT PROTOCOLS IN IMMUNOLOGY, Wiley/Greene, NY (1991); Stites (eds.) BASIC AND CLINICAL IMMUNOLOGY (7th ed.) Lange Medical Publications, Los Altos, CA ("Stites"); Goding, MONOCLONAL ANTIBODIES: PRINCIPLES AND PRACTICE (2d ed.) Academic Press, New York, NY (1986); Kohler (1975) *Nature* 256:495; Harlow (1988) ANTIBODIES, A LABORATORY MANUAL, Cold Spring Harbor Publications, New York. Such

techniques include selection of antibodies from libraries of recombinant antibodies displayed in phage ("phage display libraries") or similar on cells. *See*, Huse (1989) *Science* 246:1275; Ward (1989) *Nature* 341:544; Hoogenboom (1997) *Trends Biotechnol.* 15:62-70; Katz (1997) *Annu. Rev. Biophys. Biomol. Struct.* 26:27-45. Recombinant antibodies can be expressed by transient or stable expression vectors in mammalian cells, as in Norderhaug (1997) *J. Immunol. Methods* 204:77-87; Boder (1997) "Yeast surface display for screening combinatorial polypeptide libraries," *Nat. Biotechnol.* 15:553-557.

Purification of MBP Peptides and Complexes

The methods and reagents of the invention enable one to purify the MBP peptides and complexes of the invention from a variety of sources, depending on which natural source, synthetic or recombinant expression system selected, such as plant cells, larval homogenates, bacterial cells, yeast, mammalian cells, human cells, tissue culture media, transgenic plants and animals, to substantial purity. General information relating to standard purification procedures, is well known in the patent and scientifice literature, as described *supra*; see also, *e.g.*, Scopes, R. K., Protein Purification: Principles and Practice, 2nd ed., Springer Verlag, (1987), Banga, Ausubel, and Sambrook.

Fusion Proteins

MBP peptides and complex polypeptides can also be expressed as proteins with one or more additional polypeptide domains linked thereto to facilitate cell killing (using, *e.g.*, effector agents, as described above), protein detection, purification, or other applications. Detection and purification facilitating domains include, *e.g.*, metal chelating peptides such as polyhistidine tracts and histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle WA). The inclusion of a cleavable linker sequences such as Factor Xa or enterokinase (Invitrogen, San Diego CA) between the purification domain and plant disease resistant polypeptide may be useful to facilitate purification. For example, one expression vector includes a polypeptide-encoding nucleic acid sequence linked to six histidine residues followed by a thioredoxin and an enterokinase cleavage site (*e.g.*, see Williams (1995) *Biochemistry* 34:1787-1797). The histidine residues facilitate detection and purification while the enterokinase cleavage site provides a means for purifying the desired protein(s) from the

remainder of the fusion protein. Technology pertaining to vectors encoding fusion proteins and application of fusion proteins are well described in the scientific and patent literature, see *e.g.*, Kroll (1993) *DNA Cell. Biol.*, 12:441-53.

IV. Formulation and Administration of MBP Peptides and Complexes: Pharmaceutical Compositions

The MBP peptides and Class II:peptide complexes of the invention are typically combined with a pharmaceutically acceptable carrier (excipient) to form a pharmacological composition. Pharmaceutically acceptable carriers can contain a physiologically acceptable compound that acts to, *e.g.*, stabilize, or increase or decrease the absorption or clearance rates of the pharmaceutical compositions of the invention. Physiologically acceptable compounds can include, *e.g.*, carbohydrates, such as glucose, sucrose, or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins, compositions that reduce the clearance or hydrolysis of the peptides or polypeptide complexes, or excipients or other stabilizers and/or buffers. Detergents can also used to stabilize or to increase or decrease the absorption of the pharmaceutical composition, see *infra* for exemplary detergents, including liposomal carriers. Pharmaceutically acceptable carriers and formulations for peptides and polypeptide are known to the skilled artisan and are described in detail in the scientific and patent literature, see *e.g.*, the latest edition of Remington's Pharmaceutical Science, Mack Publishing Company, Easton, Pennsylvania ("Remington's") and Banga.

Other physiologically acceptable compounds include wetting agents, emulsifying agents, dispersing agents or preservatives which are particularly useful for preventing the growth or action of microorganisms. Various preservatives are well known and include, *e.g.*, phenol and ascorbic acid. One skilled in the art would appreciate that the choice of a pharmaceutically acceptable carrier including a physiologically acceptable compound depends, for example, on the route of administration of the protein or polypeptide of the invention and on its particular physio-chemical characteristics.

Aqueous Solutions for Enteral, Parenteral Or Transmucosal Administration

The compositions for administration will commonly comprise a solution of the peptide or polypeptide of the invention dissolved in a pharmaceutically acceptable carrier,

preferably an aqueous carrier if the composition is water-soluble. Examples of aqueous solutions that can be used in formulations for enteral, parenteral or transmucosal drug delivery include, *e.g.*, water, saline, phosphate buffered saline, Hank's solution, Ringer's solution, dextrose/saline, glucose solutions and the like. The formulations can contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as buffering agents, tonicity adjusting agents, wetting agents, detergents and the like. Additives can also include additional active ingredients such as bactericidal agents, or stabilizers. For example, the solution can contain sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate or triethanolamine oleate. These compositions can be sterilized by conventional, well-known sterilization techniques, or can be sterile filtered. The resulting aqueous solutions can be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The concentration of MBP peptide and/or Class II:peptide complex in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs.

Solid Formulations For Enteral Delivery

Solid formulations can be used for enteral (oral) administration. They can be formulated as, *e.g.*, pills, tablets, powders or capsules. For solid compositions, conventional nontoxic solid carriers can be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10% to 95% of active ingredient (MBP peptide or complex). A non-solid formulation can also be used for enteral administration. The carrier can be selected from various oils including those of petroleum, animal, vegetable or synthetic origin, *e.g.*, peanut oil, soybean oil, mineral oil, sesame oil, and the like. Suitable pharmaceutical excipients include *e.g.*, starch, cellulose, talc, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, magnesium stearate, sodium stearate, glycerol monostearate, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol, and the like.

It is recognized that the MBP peptides and polypeptide complexes of the invention, when administered orally, must be protected from digestion. This is typically accomplished either by complexing the peptide or polypeptide complex with a composition to render it resistant to acidic and enzymatic hydrolysis or by packaging the peptide or complex in an appropriately resistant carrier such as a liposome. Means of protecting compounds from digestion are well known in the art, *see, e.g.,* Fix (1996) *Pharm Res.* 13:1760-1764; Samanen (1996) *J. Pharm. Pharmacol.* 48:119-135; U.S. Patent 5,391,377, describing lipid compositions for oral delivery of therapeutic agents (liposomal delivery is discussed in further detail, *infra*).

Topical Formulations For Transdermal/ Transmucosal Delivery

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated can be used in the formulation. Such penetrants are generally known in the art, and include, *e.g.*, for transmucosal administration, bile salts and fusidic acid derivatives. In addition, detergents can be used to facilitate permeation. Transmucosal administration can be through nasal sprays or using suppositories. See, *e.g.*, Banga, Chapt. 10; Sayani (1996) "Systemic delivery of peptides and proteins across absorptive mucosae" *Crit. Rev. Ther. Drug Carrier Syst.* 13:85-184. For topical, transdermal administration, the agents are formulated into ointments, creams, salves, powders and gels. Transdermal delivery systems can also include, *e.g.*, patches. See, *e.g.*, Banga, Chapt. 9.

The peptides and polypeptide complexes can also be administered in sustained delivery or sustained release mechanisms, which can deliver the formulation internally. For example, biodegradeable microspheres or capsules or other biodegradeable polymer configurations capable of sustained delivery of a composition (*e.g.*, an MBP peptide:Class II polypeptide complex) can be included in the formulations of the invention (see, *e.g.*, Putney (1998) *Nat. Biotechnol.* 16:153-157).

Formulations for Inhalation Delivery

For inhalation, the peptide or polypeptide can be delivered using any system known in the art, including dry powder aerosols, liquids delivery systems, air jet nebulizers, propellant systems, and the like. See, *e.g.*, Patton (1998) *Biotechniques* 16:141-143; product and inhallation delivery systems for polypeptide macromolecules by, *e.g.*, Dura

Pharmaceuticals (San Diego, CA) , Aradigm (Hayward, CA), Aerogen (Santa Clara, CA), Inhale Therapeutic Systems (San Carlos, CA), and the like.

For example, the pharmaceutical formulation can be administered in the form of an aerosol or mist. For aerosol administration, the formulation can be supplied in finely divided form along with a surfactant and propellant. The surfactant preferably is soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride such as, for example, ethylene glycol, glycerol, erythritol, arabitol, mannitol, sorbitol, the hexitol anhydrides derived from sorbitol, and the polyoxyethylene and polyoxypropylene derivatives of these esters. Mixed esters, such as mixed or natural glycerides can be employed. The surfactant can constitute 0.1% to 20% by weight of the composition, preferably 0.25% to 5%. The balance of the formulation is ordinarily propellant. Liquefied propellants are typically gases at ambient conditions, and are condensed under pressure. Among suitable liquefied propellants are the lower alkanes containing up to 5 carbons, such as butane and propane; and preferably fluorinated or fluorochlorinated alkanes. Mixtures of the above can also be employed. In producing the aerosol, a container equipped with a suitable valve is filled with the appropriate propellant, containing the finely divided compounds and surfactant. The ingredients are thus maintained at an elevated pressure until released by action of the valve. See, *e.g.*, Edwards (1997) "Large porous particles for pulmonary drug delivery" *Science* 276:1868-1871.

In another embodiment, the device for delivering the formulation to respiratory tissue is an inhaler in which the formulation vaporizes. Other liquid delivery systems include, *e.g.*, air jet nebulizers.

Other Formulations

In preparing pharmaceuticals of the present invention, a variety of formulation modifications can be used and manipulated to alter pharmacokinetics and biodistribution. A number of methods for altering pharmacokinetics and biodistribution are known to one of ordinary skill in the art. Examples of such methods include protection of the complexes in vesicles composed of substances such as proteins, lipids (for example, liposomes, see below),

carbohydrates, or synthetic polymers (discussed above). For a general discussion of pharmacokinetics, see, *e.g.,* Remington's, Chapters 37-39, or Banga, Chapt. 6.

Routes of Delivery

The peptide and polypeptide complexes used in the methods of the invention can be delivered alone or as pharmaceutical compositions by any means known in the art, *e.g.*, systemically, regionally, or locally; by intraarterial, intrathecal (IT), intravenous (IV), parenteral, intra-pleural cavity, topical, oral, or local administration, as subcutaneous, intra-tracheal (*e.g.,* by aerosol) or transmucosal (*e.g.*, buccal, bladder, vaginal, uterine, rectal, nasal mucosa). Actual methods for preparing administrable compositions will be known or apparent to those skilled in the art and are described in detail in the scientific and patent literature, see *e.g.*, Remington's or Banga. Particularly preferred modes of administration include intra-arterial or intrathecal (IT) injections, especially when it is desired to have a "regional effect," e.g., to focus on a specific organ, *e.g.*, brain and CNS (see *e.g.*, Gurun (1997) *Anesth Analg.* 85:317-323). For example, intra-carotid artery injection if preferred where it is desired to deliver a peptide or polypeptide complex of the invention directly to the brain. Parenteral administration is a preferred route of delivery if a high systemic dosage is needed. Enteral administration is a preferred method if administration of peptide to induce oral tolerance is the therapeutic objective, see, *e.g.*, Kennedy (1997) *J. Immunol.* 159:1036-1044; Kent (1997) *Ann. NY Acad. Sci.* 815:412-422. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in detail, in *e.g.*, Remington's, Banga Chapt 7. See also, Bai (1997) *J. Neuroimmunol.* 80:65-75; Warren (1997) *J. Neurol. Sci.* 152:31-38; Tonegawa (1997) *J. Exp. Med.* 186:507-515.

Treatment Regimens: Pharmacokinetics

The pharmaceutical compositions can be administered in a variety of unit dosage forms depending upon the method of administration. Dosages for typical peptide and polypeptide pharmaceutical compositions are well known to those of skill in the art. Such dosages are typically advisorial in nature and are adjusted depending on the particular therapeutic context, patient tolerance, *etc.* The amount of MBP peptide or Class II:peptide complex adequate to accomplish this is defined as a "therapeutically effective dose." The dosage schedule and amounts effective for this use, *i.e.*, the "dosing regimen," will depend upon a variety of factors, including the stage of the disease or condition, the severity of the

disease or condition, the general state of the patient's health, the patient's physical status, age, pharmaceutical formulation and concentration of active agent, and the like. In calculating the dosage regimen for a patient, the mode of administration also is taken into consideration. The dosage regimen must also take into consideration the pharmacokinetics, *i.e.*, the pharmaceutical composition's rate of absorption, bioavailability, metabolism, clearance, and the like. See, *e.g.*, the latest Remington's; Egleton (1997) "Bioavailability and transport of peptides and peptide drugs into the brain" *Peptides* 18:1431-1439; Langer (1990) *Science* 249:1527-1533.

In therapeutic applications, compositions are administered to a patient suffering from a demyelinating disease in an amount sufficient to cure or at least partially arrest the disease and/or its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the disease, general state of the patient's health, frequency and routes of administration, clinician's judgement, and the like. For example, in one embodiement, a soluble Class II:peptide complex pharmaceutical composition dosage for intravenous (IV) administration would be about 0.01 mg/hr to about 1.0 mg/hr administered over several hours (typically 1, 3, or 6 hours), which can be repeated for weeks with intermittant cycles. Considerably higher dosages (*e.g.*, ranging up to about 10 mg/ml) can be used, particularly when the drug is administered to a secluded site and not into the blood stream, such as into a body cavity or into a lumen of an organ, *e.g.*, the cerebrospinal fluid (CSF).

Dosages can be be determined empirically, by assessing the abatement or amelioration of symptions, or by objective criteria, such analysis of blood or histopathology specimens. For example, in MS, the disease is characterized by various complaints and findings of CNS dysfunction, with remissions and persistently recurring exacerbations. Onset usually is insidious. The most frequent presenting symptoms paresthesias in one or more extremities, in the trunk, or on one side of the face; weakness or clumsiness of a leg or a hand; or visual disturbances (*e.g.*, partial blindness and pain in one eye, diplopia, dimness of vision, scotomas). Other common early symptoms are a fleeting ocular palsy, transient weakness of one or more extremities, slight stiffness or unusual fatigability of a limb, minor gait disturbances, difficulties with bladder control, vertigo, or mild emotional disturbances; all are evidence of scattered CNS involvement and often occurring months or years before the disease

is recognized. Successful treatment can also be monitered by histopathology. In MS, plaques or islands of demyelination with destruction of oligodendroglia and perivascular inflammation are disseminated through the CNS, primarily in the white matter, with a predilection for the lateral and posterior columns (especially in the cervical and dorsal regions), the optic nerves, and periventricular areas. Tracts in the midbrain, pons, and cerebellum also are affected, and gray matter in both cerebrum and cord may be affected. Cell bodies and axons usually are preserved, especially in early lesions. Later, axons may be destroyed, especially in the long tracts, and a fibrous gliosis gives the tracts their "sclerotic" appearance. Both early and late lesions may be found simultaneously. Thus, the compositions of the invention are administered to arrest the progress of the disease and to reduce the onset, frequency or severity of these or other symptoms.

In one exemplary embodiment, the dosage is between about 0.5 mg/kg and about 25 mg/kg, with a preferred embodiment of about 3 mg/kg to about 15 mg/kg. In another exemplary embodiment, a unit dosage is between about 0.01 to 1000 mg per dose, with a preferred embodiment of about 10 to about 100 mg per dose. See also USPN 5,468,481, issued November 21, 1995. In another embodiment, peptide is adminstered enterally to induce oral tolerance in a dosage ranging from 10 to 2500 ug per day, with a preferred embodiment of about 20 to 50 ug per day. See, *e.g.*, Barnett (1998) *Arthritis Rheum.* 41:290-297, where oral administration of cartilage-derived type II collagen (to ameliorate arthritis) at a dosage ranges of 20, 100, 500, or 2,500 ug/day showed best results using 20 ug per day.

The pharmaceutical compositions containing the peptide and complexes of the invention can be administered alone or in conjunction with other therapeutic treatments. Single or multiple administrations of the compositions may be administered depending on the dosage and frequency as required and tolerated by the patient.

Liposomal Formulations

The invention provides pharmaceuticals in which the transmembrane region of the Class II subunit of the complex is included. In one embodiment, the pharmaceutical formulations comprising this Class II:MBP peptide complex are incorporated in lipid monolayers or bilayers. The invention also provides formulations in which water soluble peptides or complexes have been attached to the surface of the monolayer or bilayer. For example, peptides can be attached to hydrazide- PEG- (distearoylphosphatidyl) ethanolamine-

containing liposomes (see, *e.g.*, Zalipsky (1995) *Bioconjug. Chem.* 6:705-708). Liposomes or any form of lipid membrane, such as planar lipid membranes or the cell membrane of an intact cell, *e.g.*, a red blood cell, can be used. Liposomal formulations can be by any means, including administration intravenously, transdermally (see, *e.g.*, Vutla (1996) *J. Pharm. Sci.* 85:5-8), transmucosally, or orally. The invention also provides pharmaceutical preparations in which the peptides and/or complexes of the invention are incorporated within micelles and/or liposomes (see, *e.g.*, Suntres (1994) *J. Pharm. Pharmacol.* 46:23-28; Woodle (1992) *Pharm. Res.* 9:260-265).

Liposomes and liposomal formulations can be prepared according to standard methods and are also well known in the art, see, *e.g.*, Remington's; Akimaru (1995) *Cytokines Mol. Ther.* 1:197-210; Alving (1995) *Immunol. Rev.* 145:5-31; Szoka (1980) *Ann. Rev. Biophys. Bioeng.* 9:467, U.S. Pat. Nos. 4, 235,871, 4,501,728 and 4,837,028. In one embodiment, liposomes of the present invention typically contain the Class II:peptide complexes positioned on the surface of the liposome in such a manner that the complexes are available for interaction with the TCR. The transmembrane region is usually first incorporated into the membrane at the time of forming the membrane. The liposomes can also be used to target desired drugs (*e.g.* toxins or chemotherapeutic agents) to particular autoreactive T cells.

Liposome charge is an important determinant in liposome clearance from the blood, with negatively charged liposomes being taken up more rapidly by the reticuloendothelial system (Juliano (1975) *Biochem. Biophys. Res. Commun.* 63:651) and thus having shorter half-lives in the bloodstream. Incorporating phosphatidylethanolamine derivatives enhance the circulation time by preventing liposomal aggregation. For example, incorporation of N-(omega-carboxy)acylamido-phosphatidylethanolamines into large unilamellar vesicles of L-alpha-distearoylphosphatidylcholine dramatically increases the *in vivo* liposomal circulation lifetime (see, *e.g.*, Ahl (1997) *Biochim. Biophys. Acta* 1329:370-382). Liposomes with prolonged circulation half-lives are typically desirable for therapeutic and diagnostic uses. For instance, liposomes which can be maintained from 8, 12, or up to 24 hours in the bloodstream are particularly preferred embodiments of the invention.

Typically, the liposomes are prepared with about 5 to 15 mole percent negatively charged phospholipids, such as phosphatidylglycerol, phosphatidylserine or phosphatidyl-inositol. Added negatively charged phospholipids, such as phosphatidylglycerol,

also serve to prevent spontaneous liposome aggregating, and thus minimize the risk of undersized liposomal aggregate formation. Membrane-rigidifying agents, such as sphingomyelin or a saturated neutral phospholipid, at a concentration of at least about 50 mole percent, and 5 to 15 mole percent of monosialylganglioside, may provide increased circulation of the liposome preparation in the bloodstream, as generally described in USPN 4, 837,028.

Additionally, the liposome suspension can include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as alpha-tocopherol and water-soluble iron-specific chelators, such as ferrioxianine, are preferred.

The formulations of the invention can include multilamellar vesicles of heterogeneous sizes. In this method, the vesicle-forming lipids are dissolved in a suitable organic solvent or solvent system and dried under vacuum or an inert gas to form a thin lipid film. If desired, the film may be redissolved in a suitable solvent, such as tertiary butanol, and then lyophilized to form a more homogeneous lipid mixture which is in a more easily hydrated powderlike form. This film is covered with an aqueous solution of the peptide or polypeptide complex and allowed to hydrate, typically over a 15 to 60 minute period with agitation. The size distribution of the resulting multilamellar vesicles can be shifted toward smaller sizes by hydrating the lipids under more vigorous agitation conditions or by adding solubilizing detergents such as deoxycholate. The hydration medium contains the peptide or complex at a concentration which is desired in the interior volume of the liposomes in the final liposome suspension. Typically the drug solution contains between 10 to 100 mg/ml of the peptides or complexes of the invention in a buffered saline solution.

Following liposome preparation, the liposomes can be sized to achieve a desired size range and relatively narrow distribution of liposome sizes. One preferred size range is about 0.2 to 0.4 microns, which allows the liposome suspension to be sterilized by filtration through a conventional filter, typically a 0.22 micron filter. The filter sterilization method can be carried out on a high through-put basis if the liposomes have been sized down to about 0.2 to 0.4 microns. Several techniques are available for sizing liposome to a desired size (see, *e.g.*, U.S. Pat. No. 4,737,323). Sonicating a liposome suspension either by bath or probe sonication produces a progressive size reduction down to small unilamellar vesicles less than about 0.05 microns in size. Homogenization is another method which relies on shearing

energy to fragment large liposomes into smaller ones. In a typical homogenization procedure, multilamellar vesicles are recirculated through a standard emulsion homogenizer until selected liposome sizes, typically between about 0.1 and 0.5 microns, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size discrimination. Extrusion of liposome through a small-pore polycarbonate membrane or an asymmetric ceramic membrane is also an effective method for reducing liposome sizes to a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired liposome size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size.

Even under the most efficient encapsulation methods, the initial sized liposome suspension may contain up to 50% or more complex in a free (nonencapsulated) form. Several methods are available for removing non-entrapped compound from a liposome suspension, if desired for a particular formulation. In one method, the liposomes in the suspension are pelleted by high-speed centrifugation leaving free compound and very small liposomes in the supernatant. Another method involves concentrating the suspension by ultrafiltration, then resuspending the concentrated liposomes in a replacement medium. Alternatively, gel filtration can be used to separate large liposome particles from solute molecules. Following this treatment, the liposome suspension can be brought to a desired concentration for use in, *e.g.*, an intravenous, I.P, transdermal, or transmucosal administration. This involve resuspending the liposomes in a suitable volume of appropritate medium, where the liposomes have been concentrated, for example by centrifugation or ultrafiltration, or concentrating the suspension, where the drug removal step has increased total suspension volume. The suspension is then sterilized by filtration as described above. These liposomes comprising the peptides or Class II:peptide complex can be administered parenterally or locally in a dose which varies according to, *e.g.*, the manner of administration, the drug being delivered, the particular disease being treated, etc.

Micelles are commonly used in the art to increase solubility of molecules having nonpolar regions. One of skill will thus recognize that micelles are useful in compositions of the present invention. Micelles comprising the complexes of the invention are prepared according to methods well known in the art (see, *e.g.*, Remington's, Chap. 20).

Micelles comprising the peptides and/or complexes of the present invention are typically prepared using standard surfactants or detergents. Micelles are formed by surfactants (molecules that contain a hydrophobic portion and one or more ionic or otherwise strongly hydrophilic groups) in aqueous solution. As the concentration of a solid surfactant increases, its monolayers adsorbed at the air/water or glass/water interfaces become so tightly packed that further occupancy requires excessive compression of the surfactant molecules already in the two monolayers. Further increments in the amount of dissolved surfactant beyond that concentration cause amounts equivalent to the new molecules to aggregate into micelles. Suitable surfactants include sodium laureate, sodium oleate, sodium lauryl sulfate, octaoxyethylene glycol monododecyl ether, octoxynol 9 and PLURONIC F-127® (Wyandotte Chemicals Corp.). Preferred surfactants are nonionic polyoxyethylene and polyoxypropylene detergents compatible with IV injection such as PLURONIC F-127®, n-octyl-alpha-D-glucopyranoside, and the like. Phospholipids, such as those described for use in the production of liposomes, can also be used for micelle formation. Since, in some embodiments of the invention, the Class II subunit of the complexes comprise a lipophilic transmembrane region and a relatively hydrophilic extracellular domain, mixed micelles can be formed in the presence of common surfactants or phospholipids and the subunits. The mixed micelles of the present invention can comprise any combination of the subunits, phospholipids and/or surfactants. Thus, the micelles can comprise subunits and detergent, subunits in combination with both phospholipids and detergent, or subunits and phospholipid.

Kits can also be supplied for therapeutic or diagnostic uses. In one embodiment the pharmaceutical formulation of the invention is in a lyophilized form, which can be placed in a container. The complexes, which can also be conjugated to a label or toxin, or unconjugated, are included in the kits with buffers, such as Tris, phosphate, carbonate, etc., stabilizers, biocides, inert proteins, *e.g.*, serum albumin, or the like, and a set of instructions for use. Generally, these materials will be present in less than about 5% wt. based on the amount of complex and usually present in total amount of at least about 0.001% wt. based again on the protein concentration. Frequently, it will be desirable to include an inert extender or excipient to dilute the active ingredients, where the excipient may be present in from about 1% to 99% wt. of the total composition. Where an antibody capable of binding to the complex

is employed in an assay, this will usually be present in a separate vial. The antibody is typically conjugated to a label and formulated according to techniques well known in the art.

DEFINITIONS

To facilitate understanding the invention, a number of terms are defined below.

The term "antibody" refers to a polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments or synthetic or recombinant analogues thereof which specifically bind and recognize analytes and antigens, such as a genus or subgenus of polypeptides of the invention, as described *supra*.

The term "conservative substitution" refers to a change in the amino acid composition of a peptide or protein, such as polypeptide comprising the MBP peptide sequences of the invention, that does not substantially alter the peptide's or protein's activity. This includes conservatively modified variations of a particular amino acid sequence, *i.e.*, amino acid substitutions of those amino acids that are not critical for protein activity or substitution of amino acids with other amino acids having similar properties (*e.g.*, acidic, basic, positively or negatively charged, polar or non-polar, *etc.*) such that the substitutions of even critical amino acids does not substantially alter activity. A polypeptide sequence of the invention implicitly encompasses conservatively substituted variants thereof. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following six groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (a), Serine (S), Threonine (T); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W) (see also, Creighton (1984) *Proteins*, W.H. Freeman and Company). One of skill in the art will appreciate that the above-identified substitutions are not the only possible conservative substitutions. For example, for some purposes, one may regard all charged amino acids as conservative substitutions for each other whether they are positive or negative. In addition, individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids in an encoded sequence can also be considered "conservatively modified variations." The term "conservative substitution" also refers to a change in a nucleic acid sequence such that the substitution does not substantially alter the

contemplated activity of the nucleic acid, for example, as not changing the activity of the peptide or protein encoded by the nucleic acid. A nucleic acid sequence of the invention implicitly encompasses conservatively modified variants thereof (*e.g.* degenerate codon substitutions) and complementary sequences and as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer (1991) *Nucleic Acid Res.* 19:5081; Ohtsuka (1985) *J. Biol. Chem.* 260:2605-2608; Rossolini (1994) *Mol. Cell. Probes* 8:91-98).

A "fusion protein" refers to a composition comprising at least one polypeptide or peptide domain which is associated with a second domain. The second domain can be a polypeptide, peptide, polysaccharide, or the like. The "fusion" can be an association generated by a chemical linking or by a charge (electrostatic attraction, *i.e.*, salt bridges, H-bonding, etc.) interaction. If the polypeptides are recombinant, the "fusion protein" can be translated from a common message. Alternatively, the compositions of the domains can be linked by any chemical or electrostatic means.

A "heterologous sequence" refers to any amino acid or nucleic acid sequence that is not a myelin basic protein sequence.

As used herein, "isolated," when referring to a molecule or composition, such as, for example, an MBP peptide, Class II:peptide complex, or nucleic acid, means that the molecule or composition is separated from at least one other compound, such as a protein, other nucleic acids (*e.g.*, RNAs), or other contaminants with which it is associated *in vivo* or in its naturally occurring state. Thus, a polypeptide or nucleic acid is considered isolated when it has been isolated from any other component with which it is naturally associated, *e.g.*, cell membrane, as in a cell extract. An isolated composition can, however, also be substantially pure. An isolated composition can be in a homogeneous state and can be in a dry or an aqueous solution. Purity and homogeneity can be determined, for example, using analytical chemistry techniques such as polyacrylamide gel electrophoresis (SDS-PAGE) or high performance liquid chromatography (HPLC).

The term "nucleic acid" or "nucleic acid sequence" refers to a deoxyribonucleotide or ribonucleotide oligonucleotide in either single- or double-stranded form. The term encompasses nucleic acids, *i.e.*, oligonucleotides, containing known analogues

of natural nucleotides which have similar or improved binding properties, for the purposes desired, as the reference nucleic acid. The term also includes nucleic acids which are metabolized in a manner similar to naturally occurring nucleotides or at rates that are improved thereover for the purposes desired. The term also encompasses nucleic-acid-like structures with synthetic backbones. DNA backbone analogues provided by the invention include phosphodiester, phosphorothioate, phosphorodithioate, methylphosphonate, phosphoramidate, alkyl phosphotriester, sulfamate, 3'-thioacetal, methylene(methylimino), 3'-N-carbamate, morpholino carbamate, and peptide nucleic acids (PNAs); see Oligonucleotides and Analogues, a Practical Approach, edited by F. Eckstein, IRL Press at Oxford University Press (1991); Antisense Strategies, Annals of the New York Academy of Sciences, Volume 600, Eds. Baserga and Denhardt (NYAS 1992); Milligan (1993) *J. Med. Chem.* 36:1923-1937; Antisense Research and Applications (1993, CRC Press). PNAs contain non-ionic backbones, such as N-(2-aminoethyl) glycine units. Phosphorothioate linkages are described in WO 97/03211; WO 96/39154; Mata (1997) *Toxicol Appl Pharmacol* 144:189-197. Other synthetic backbones encompasses by the term include methyl-phosphonate linkages or alternating methylphosphonate and phosphodiester linkages (Strauss-Soukup (1997) *Biochemistry* 36:8692-8698), and benzylphosphonate linkages (Samstag (1996) *Antisense Nucleic Acid Drug Dev* 6:153-156). The term nucleic acid is used interchangeably with gene, cDNA, mRNA, oligonucleotide primer, probe and amplification product.

The term "pharmaceutical composition" refers to a composition suitable for pharmaceutical use in a subject. The pharmaceutical compositions of this invention are formulations that comprise a pharmacologically effective amount of an MBP peptide and/or a Class II:peptide complex and a pharmaceutically acceptable carrier.

The terms "to treat" and "treatment" refer to prophylactic and therapeutic treatment administered to a subject. Prophylactic treatment includes administering treatment to an individual who does not have a disease or condition, or does not exhibit signs of a disease or condition, or exhibits only early signs of a disease or condition for the purpose of decreasing or abrogating the risk of acquiring the disease or condition or decreasing or abrogating a symptom or a pathologic condition arising from or related to the disease or pathology. The invention provides a method to treat a T cell mediated immune response

against myelin basic protein in a subject by administration of an MBP peptide and/or a Class II:peptide complex to an individual who is considered a high risk for developing a demyelinating disease or condition. Therapeutic treatment also includes administering treatment to an individual who exhibits signs of pathology or disease, or is considered at risk for developing or incurring such a condition or disease, for the purpose of preventing, diminishing, ameliorating or eliminating any symptoms or pathology. Thus, the methods of the invention provide a means to treat demyelinating disease and to prevent, diminish, eliminate or otherwise ameliorate the disease's clinical symptoms and tissue pathology, as discussed above. A "pharmacologically effective amount" is the amount of compound administered to the individual to prophylactically or therapeutically effectively treat an individual for a disease or condition.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

EXAMPLES

The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1 Identification of core structure and critical TCR contact residues in an antigenic MBP peptide by measuring T cell activation signals

The invention provides novel MBP peptides and MPB peptide:Class II complexes, pharmaceutical formulations comprising these compositions, and a method for treating a T cell mediated immune response against MPB in a subject by administration of these novel compositions. The following example details the identification of novel MBP peptides which include the core TCR recognition sequence amino acid residues involved in the binding of autoreactive TCR associated with demyelinating disease, as MS, with MBP peptide and an appropriate Class II molecule.

A novel, minimal structure and critical residues for its interaction with autoreactive TCR was identified for human myelin basic protein (MBP) peptide. Synthetic

peptide analogs were incubated with TCRs on a DRB5*0101-restricted transformed human T cell clone (SS8T) expressing MHC class II (DR2) molecules. A silicon-based biosensor microphysiometer was used to measure the real time cell response to a T cell activation event stimulated by the binding of Class II:peptide to TCR. The microphysiometer monitored changes in extracellular acidification rate in response to MBP peptide:Class II polypeptide binding to autoreactive TCRs. Increases in the extracellular acidification rate is a direct result of early T cell signaling events, such as high affinity TCR binding to a Class II:peptide complex. Cultured SS8T cells were exposed to N- (amino)-terminus and C- (carboxy)-terminus truncated MBP peptides separately in microphysiometer chambers to determine the minimal amino acid residues required for the T cell response. In parallel, analogs of MBP peptide with single alanine substitutions were tested in this assay to identify critical amino acid residues involved in TCR interactions. As described below, a minimal core length of ten amino acid residues was determined to correspond to peptide MBP(91-100) (SEQ ID NO:38) and residues F-91, K-93, N-94, I-95 and V-96 were determined to be essential for TCR interaction. Acidification rate measurements correlated well with enhanced levels of gamma-interferon (IFN) and tumor necrosis factor (TNF)-alpha cytokine production.

Herpes virus saimiri (HVS) immortalized human T cells were used in *in vitro* assays to identify the novel MBP peptides of the invention. HVS T cells express the same level of TCR as normal human T cells, without any loss of antigen recognition (Weber (1993) *Proc. Natl. Acad. Sci. USA* 90:11049). Specifically, the HVS transformed human T cell clone used in these assays, designated SS8T, was generated from an MS patient. SS8T has previously been characterized as being restricted to the HLA Class II DR2(DRB5*0101) polypeptide and the MBP(84-102) peptide (Weber (1993) *supra*). A modified peptide analog of MBP(84-102) with a N-acetylated tyrosine residue at the N-terminus (Ac-MBP(83-102)Y^{83}) has been characterized as being recognized by SS8T transformed T cells when complexed with HLA DR2 (DRB5*0101) (Mukku (1995) *Mol. Immunol.* 32:555; Arimilli (1995) *J. Biol. Chem.* 270:971). To identify the minimum length and critical TCR contact residues of this MBP(83-102)Y^{83} peptide, different terminal truncated and alanine analog peptides were synthesized. The various peptides used in this study are presented schematically in Fig. 1A and Fig 1B. For alanine analog peptides, a single amino acid was substituted with alanine for each residue in the MBP(90-102)Y^{83} sequence.

The critical MBP peptide amino acid residues required for TCR engagement in this model system depend on two factors; the peptide residues responsible for binding to MHC class II binding groove amino acid residues (the peptide agretope) and the effective interaction of some of the amino acid residues of the peptide with TCR (the epitope). To determine the minimum length of the immunodominant epitope, various terminally truncated peptides were incubated at different concentrations with SS8T cells in a microtiter plate for 48 hours at 37°C. After incubating the T cells with peptide, culture fluids were tested for the presence of secreted gamma IFN and TNF-beta by ELISA using monoclonal antibodies (mAbs) to each (anti-human gamma IFN mAb and rabbit anti-human gamma IFN polyclonal Ab, Endogen Inc., Woburn, MA; peroxidase-conjugated goat IgG and rabbit IgG, Jackson Immunoresearch Laboratories, West Grove, PA; human gamma IFN, Boehringer Mannheim, Indianapolis, IN; anti-human TNF-beta mAb, goat anti-human TNF-beta polyclonal and recombinant human TNF-beta, R&D Systems Inc, Minneapolis, MN). In parallel analyses, SS8T cells were immobilized in agarose and exposed to various terminal truncated peptides in the microphysiometer chamber to monitor their extracellular acidification rates.

Peptides prepared for these studies were acetylated at the N (amino)-terminus and amidated at the C (carboxy)-terminus. The MBP(83-102) peptide with the sequence Ac-YDENPVVHFFKNIVTPRTPP (SEQ ID NO:1); and the MBP(124-143) peptide with the sequence Ac-GFGYGGRASDYKSAHKGFKG (SEQ ID NO:37) were synthesized by the standard solid phase method using side-chain protected Fmoc amino acids on an Applied Biosystems 431A automated peptide synthesizer. The deprotected, crude peptides were purified by reverse-phase HPLC, and the homogeneity and identity of the purified peptides were confirmed by mass spectrometry. All terminally truncated and alanine analog MBP peptides (Fig. 1) were synthesized by solid phase peptide synthesis using Fmoc chemistry. All chemicals, including the Rink amide MBRA resin and side chain protected Fmoc amino acids were obtained from Nova Biochem, San Diego, CA. HBTU/HOBt or PyBOP activation chemistry were employed for protected amino acid coupling on ABI 431 A automated peptide synthesizer or ABIMED/GILSON AMS 422 multiple peptide synthesizer, as described by Luu (1996) *Int. J. Peptide Protein Res.* 47:91. After peptides were synthesized on solid phase, they were cleaved by TFA containing 5% 4-methoxybenzenethiol and 5% 4-methylmercaptophenol as scavengers. The crude peptides were precipitated by pentane:acetone mixture (4:1, v:v) and

isolated by centrifugation. Peptides were washed with pentane:acetone mixture three times followed by pentane and were dried in vacuum. Peptides were purified by reverse phase HPLC using a C18 column; the pure fractions were pooled and lyophilized. Peptides were characterized by electron spray mass spectrometry.

T cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 ug/ml streptomycin, 10% fetal bovine serum and 50 units/ml human recombinant IL-2 (rIL-2) at 37°C. Every alternate day, cells were transferred to fresh culture medium. Various peptides at different concentrations were incubated with the cells in a microtiter tissue culture plate at a density of 20,000 cells/200 ul/well in the absence of rIL-2. After 48 h of incubation at 37°C, the culture fluids were collected from each well to test for gamma-IFN and TNF-beta cytokines. The detection of gamma-IFN was by Ab ELISA as described by Arimilli (1995) *supra*. For the detection of TNF-beta, Nunc Maxisorb 96-well plates were coated with anti-human TNF-beta mAb at a concentration of 0.5 ug/well and incubated at 4°C overnight. The wells were blocked with 0.1% bovine serum albumin, and samples were incubated at RT for 2 h. A standard curve was generated by using recombinant human TNF-beta with a dilution range of 500 to 0.01 ng/ml. Goat anti-human TNF-beta was then added at a concentration of 1 ug/ml and plates were incubated at 25°C for an additional 2 h. Wells were washed three times and incubated with HRP-conjugated mouse anti-goat Ab at a concentration of 1 ug/ml for 1 h at 21°C, prior to developing color using 3,3',5,5'-tetramethyl benzidine (TMB, Moss, Inc, Pasadena, MD) as a substrate. The reaction was stopped by 2N sulfuric acid at 5 min, and the absorbance was measured at 450 nm.

Freshly cultured SS8T cells were immobilized into microphysiometer cell capsules as described by Nag et al., 1992, using low-melt agarose (Molecular Devices Corp., Sunnyvale, CA). Briefly, T cells were rested from IL-2 pulsing for 2 days. Cells were counted and suspended in a serum-free loading medium (low-buffering RPMI 1640 containing 10% fatty acid free, endotoxin-free, BSA). Cells were collected by centrifugation and resuspended at a concentration of 3 x 10^5 T cells per 7.5 ul of loading medium. Low-melt agarose, melted and stored at 37°C, was added to the suspended cells to a concentration of 2.5 ul per 7.5 ul. Ten ul of the agarose/cell mixture was immediately spotted into the center of the cell capsule cups (Molecular Devices Corp.) held in a 12 well culture plate. After 5 minutes 2 ml of loading medium was placed in the capsule cup over the solidified agarose and

a membrane insert was placed over the cells. The assembled cell capsule was loaded in the Cytosensor chamber at 37°C and perfused at 50 ul per min with low-buffering RPMI 1640 medium containing 10% of BSA per ml but no added HEPES or bicarbonate. Extracellular acidification measurements were made in the Cytosensor microphysiometer as described by McConnell (1992) *Science* 257:1906, by collecting potentiometric measurements for 45 seconds every 2 min. Acidification rate data (uV/sec) were normalized to 100% prior to cell stimulation, which allowed for comparison of data from cells in separate chambers.

Figure 2A, 2B and 2C show the results of these analyses, demonstrating increased levels of gamma-IFN, TNF-beta and extracellular acidification rates with first six N-terminal truncated peptides: MBP(84-102) (SEQ ID NO:2), MBP(85-102) (SEQ ID NO:3), MBP(86-102) (SEQ ID NO:4), MBP(87-102) (SEQ ID NO:5), MBP(88-102) (SEQ ID NO:6), and MBP(89-102) (SEQ ID NO:7). The SS8T cells exposed to these peptides show a 2 to 4 fold increase in gamma IFN and a 3 to 4 fold increase in TNF-beta production in a dose dependent manner (Fig. 2A, 2B). Similarly, increase in acidification rates at 118% of basal rate were observed within 10 min (Fig. 2C), indicating that the amino acid residue 84, residue 85, residue 86, residue 87, residue 88, and residue 89 are not important for TCR recognition in SS8T cells. The gamma-IFN and TNF-beta cytokine concentrations measured after 48 hour (h) correlated well with the acidification rates.

Figure 2D, 2E and 2F show results comparing the gamma-IFN, TNF-beta and acidification rates of next five N-terminus truncated MBP(90-102) (SEQ ID NO:8), MBP(91-102) (SEQ ID NO:9), MBP(92-102) (SEQ ID NO:10), MBP(93-102) (SEQ ID NO:11), and MBP(94-102) (SEQ ID NO:12) peptides. Among them the truncation of amino acid residue 91, residue 92, and residue 93 did not produce gamma-IFN, TNF-beta cytokines and acidification rates. This demonstrates that these residues are critical in T cell stimulation. Since, in one study, residue 92 (F-92) was suggested to be important for binding to Class II (Wucherpfennig (1994) *J. Exp. Med.* 179:279). The absence of T cell response by Class II:MBP(93-102) (SEQ ID NO:11) complex could be due to lack of peptide binding to class II polypeptide or lack of critical TCR contact residues (epitope residues).

In a separate assay, SS8T cells were incubated with six C-terminal truncated peptides MBP(83-101) (SEQ ID NO:13), MBP(83-100) (SEQ ID NO:14), MBP(83-99) (SEQ ID NO:15), MBP(83-98) (SEQ ID NO:16), MBP(83-97) (SEQ ID NO:17) and MBP(83-96)

(SEQ ID NO:18). As shown in Fig. 3A, 3B, and 3C, only the parent sequence (SEQ ID NO:1) and MBP(83-101) (SEQ ID NO:13) showed an increase in gamma-IFN, TNF-beta or acidification rates. This demonstrates that that C-terminal amino acid residue 100, residue 99, residue 98, residue 97 and residue 96 are important in TCR stimulation by MBP. Small quantities of gamma-IFN and TNF-beta were produced with MBP(83-100) (SEQ ID NO:14) and MBP(83-99) (SEQ ID NO:15) peptides. Similar analysis of the additional five C-terminal truncated peptides MBP(83-95) (SEQ ID NO:19), MBP(83-94) (SEQ ID NO:20), MBP(83-93) (SEQ ID NO:21), MBP(83-92) (SEQ ID NO:22), and MBP(83-91) (SEQ ID NO:23), did not show either stimulation of gamma-IFN, TNF-beta or an increase in acidification rate (Fig. 3D, 3E and 3F). These data demonstrate that amino acid residue 92, residue 93, residue 94 and residue 95 are important in T cell recognition of the peptide. The results of both N-terminal and C-terminal truncation demonstrate that FFKNIVTPRT (MBP(91-100) (SEQ ID NO:38)) is the minimal core sequence for TCR stimulation by MBP when presented by Class II DRB5*0101.

The contribution of individual amino acids in a peptide towards TCR engagement can be studied by various methods, including the D-amino acid analogs, substitution of conserved amino acid residues, switching of charge on the amino acids, and alanine substitution. Alanine substitution is more popular since it is the simplest side chain amino acid with a chiral center, and it creates only minor disturbances in the secondary structure of polypeptides and proteins. It is a common replacement for all but aromatic amino acids, based on comparison of evolutionary related proteins. Therefore, an alanine substitution strategy was used to define important TCR contact residues. Various analog peptides from MBP(90-102) (SEQ ID NO:8) with single amino acid substitutions using alanine were incubated with SS8T cells. The culture fluids were tested for gamma-IFN, TNF-beta and compared with acidification rates. Figures 4A, 4B and 4C show that two peptides, MBP(83-102)A90 (SEQ ID NO:24) and MBP(83-102)A92 (SEQ ID NO:26), were able to induce gamma-IFN, TNF-beta along with increased acidification rates when presented to SS8T cells by Class II DRB5*0101. MBP(83-102)A91 (SEQ ID NO:25), MDP(83-102)A93 (SEQ ID NO:27), MDP(83-102)A94 (SEQ ID NO:28), and MDP(83-102)A95 (SEQ ID NO:29), peptides failed to induce cytokines and showed no increase in acidification rates. This demonstrates that the amino acid residues F-91, K-93, N-94, and I-95 are important in

T cell stimulation. In one study, residue F-91 and K-93 were suggested to be secondary and N-94 to be the primary TCR contact residues in the MBP(84-102) peptide (Vergelli (1997) *J. Immunol.* 158:3746). The instant data demonstrates that residues F-91, K-93, N-94, and I-95 are important TCR contact residues in the MBP(84-102) peptide in this system.

The alanine analogs MBP(83-102)A97 (SEQ ID NO:31), MBP(83-102)A98 (SEQ ID NO:32), MBP(83-102)A99 (SEQ ID NO:33), MBP(83-102)A100 (SEQ ID NO:34), MBP(83-102)A101 (SEQ ID NO:35), and MBP(83-102)A102 (SEQ ID NO:36) show increasing levels of gamma-IFN, TNF-beta cytokines along with increased acidification rates. Only MBP(83-102)A96 (SEQ ID NO:30) failed to induce cytokines and showed no increase in acidification rates (Fig. 4D, 4E and 4F). This experiment demonstrates that V-96 is also an important residue for T cell recognition. As shown in Figure 4F, except for the peptide MDP(83-102)A96 (SEQ ID NO:30), all peptides show an immediate increase in acidification rate within 10 min. Thus, the acidification rates with alanine analog peptides was correlated with the gamma-IFN and TNF-beta production by SS8T cells. In another study, the residues N-94 and V96 were suggested to be TCR contact residues for a DRB5*0101 and MBP(84-102) peptide restricted T cell clone (Wucherpfennig (1994) *supra*).

In all experiments, MBP(124-143) (SEQ ID NO:37) was used as a negative control. MBP(124-143) peptide has a high affinity for binding to DR2 (Mukku (1995) *supra*), but does not stimulate DRB5*0101 restricted T cells (Arimilli (1995) *supra*).

Figure 5A highlights (boxed area) the core TCR recognition sequence as MBP(91-100) (SEQ ID NO:38). As described above, this peptide was identified by measuring extracellular acidification rates and by the gamma-IFN, TNF-beta cytokine response in SS8T cells using terminally truncated peptides. Figure 5B indicates (by arrows) the important amino acid residues involved in TCR contact as F-91, K-93, N-94, I-95, and V-96. As described above, these amino acids were identified from extracellular acidification rates and gamma-IFN, TNF-beta cytokine response in SS8T cells by using alanine analog peptides.

Studies were performed to demonstrate a requirement for the peptides to bind Class II polypeptide prior to T cell stimulation, *i.e.*, to determine whether T cell stimulation is the direct consequence of TCR engagement through MHC-peptide complex formed on the cell surface or due to the direct binding of the peptide to the TCR. Ab blocking experiments were performed using anti-TCR mAb and anti-Class II mAb (hybridoma cell line L243,

producing mAb to monomorphic human HLA DR molecules was obtained from American Type Culture Collection, Bethesda, MD). Transformed SS8T human T cells bear Class II on their surface, confirmed by flow cytometry. SS8T cells were incubated with either anti-TCR or anti-DR Ab to block the TCR and Class II sites. The cells were then washed and loaded in the microphysiometer chambers and exposed to 10 ug/ml MBP(83-102) (SEQ ID NO:1) peptide. Blocking TCR or Class II with specific mAb resulted in complete inhibition in acidification rate increase. These results also demonstrate that peptide binding to Class II molecules on a cell surface is a rapid cell activation phenomenon (McConnell (1995) *Proc. Natl. Acad. Sci. USA* 92:2750). After the high affinity binding of TCR with peptide:Class II polypeptide complex, early cell activation events (signals), as measured by extracellular acidification by microphysiometer, occur within 10 min. Later cell activation events (signals) are measured within 48 hours; representative later activation events measured in these studies were the gamma-IFN and TNF-beta cytokine response. Measurement of early and late activation events are equivalent in identifying peptides that are important for TCR recognition, as was done to identify the novel MBP peptides of the invention.

In summary, these studies have identified a novel family of MBP peptides characterized by having an amino acid sequence Phe -X - Lys - Asn - Ile - Val - X - X - X - Thr - X -X, wherein X is any amino acid. These peptides are also included in novel Class II:MBP complexes of the invention. These complexes comprise an MHC Class II complex capable of binding a T cell receptor, the complex consisting essentially of: an MHC Class II polypeptide comprising an extracellular domain of an MHC Class II molecule sufficient to form an antigen binding pocket and a myelin basic protein having an amino acid sequence Phe -X - Lys - R_1 - Ile - Val - X - X - X - Thr - X -X , wherein X is any amino acid, and R_1 is Asn or Gln; wherein the MBP peptide is bound to the MHC Class II component antigen binding pocket.

WHAT IS CLAIMED IS:

1. An isolated myelin basic protein (MBP) peptide, said peptide characterized by having an amino acid sequence Phe -X - Lys - Asn - Ile - Val - X - X - X - Thr - X -X, wherein X is any amino acid.

2. The isolated myelin basic protein peptide of claim 1, said peptide characterized by having an amino acid sequence Phe - Phe - Lys - Asn - Ile - Val - Thr - Pro - Arg - Thr - Pro - Pro, or a conservative substitution thereof.

3. The isolated myelin basic protein peptide of claim 1, which is linked to a heterologous sequence.

4. The isolated myelin basic protein peptide of claim 3, which is a fusion protein.

5. An isolated myelin basic protein peptide which specifically binds to an antibody directed against a peptide characterized by having an amino acid sequence Phe - Phe - Lys - Asn - Ile - Val - Thr - Pro - Arg - Thr - Pro - Pro.

6. An isolated nucleic acid encoding a myelin basic protein peptide, said peptide characterized by having an amino acid sequence Phe - X - Lys - Asn - Ile - Val - X - X - X - Thr - X - X , wherein X is any amino acid.

7. The isolated nucleic acid of claim 6, wherein the encoded myelin basic protein peptide is characterized by having an amino acid sequence Phe - Phe - Lys - Asn - Ile - Val - Thr - Pro - Arg - Thr - Pro - Pro , or a conservative substitution thereof.

8. The isolated nucleic acid of claim 6, wherein the nucleic acid comprises SEQ ID NO:1.

9. A composition comprising an MHC Class II complex capable of binding a T cell receptor, the complex consisting essentially of:

an MHC Class II polypeptide comprising an extracellular domain of an MHC Class II molecule sufficient to form an antigen binding pocket, said component being encoded by an allele associated with an autoimmune disease directed to myelin basic protein, wherein the Class II component is soluble under physiological conditions in the absence of detergent or lipid; and,

a myelin basic protein having an amino acid sequence Phe -X - Lys - R_1 - Ile - Val - X - X - X - Thr - X -X , wherein X is any amino acid, and R_1 is Asn or Gln;

wherein the myelin basic protein peptide is bound to the MHC Class II component antigen binding pocket.

10. The composition of claim 9, wherein the myelin basic protein peptide has an amino acid sequence Phe - Phe - Lys - Asn - Ile - Val - Thr - Pro - Arg - Thr - Pro - Pro or a conservative substitution thereof.

11. The composition of claim 9, which is a fusion protein.

12. The composition of claim 9, further comprising an effector composition.

13. The composition of claim 9, wherein the autoimmune disease directed to myelin basic protein is multiple sclerosis.

14. The composition of claim 9, wherein the Class II polypeptide comprises the antigen binding pocket of an HLA DR2.

15. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a pharmacologically effective amount of a peptide characterized by having an amino acid sequence Phe -X - Lys - Asn - Ile - Val - X - X - X - Thr - X -X, wherein X is any amino acid.

16. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a pharmacologically effective amount of the composition of claim 9.

17. An antibody, specifically immunoreactive under immunologically reactive conditions, to a myelin basic protein peptide, said peptide characterized by having an amino acid sequence Phe - Phe - Lys - Asn - Ile - Val - Thr - Pro - Arg - Thr - Pro - Pro.

18. An antibody, specifically immunoreactive under immunologically reactive conditions, to a myelin basic protein peptide comprising the peptide encoded by the nucleic acid of claim 6.

19. A method for inhibiting a T cell mediated immune response against myelin basic protein in a subject, comprising administering to the subject the peptide of claim 1 or the composition of claim 9 in an amount effective to treat the T cell mediated immune response.

20. The method of claim 17, wherein the T cell mediated immune response causes a pathology to a neurological system.

21. The method of claim 17, wherein the pathology to the neurological system is characterized as multiple sclerosis.

22. A method of identifying a T cell epitope on an antigen which, when bound to the antigen binding pocket of an MHC Class II molecule, is capable of binding to a T cell receptor, such binding triggering an extracellular acidification reaction by a T cell expressing the T cell receptor, said method comprising the steps of:

a) providing a composition comprising the T cell epitope bound to the antigen binding pocket of an MHC Class II molecule;

b) contacting a T cell expressing the T cell receptor with the epitope; and

c) measuring the extracellular acidification, wherein a change in the extracellular acidification indicates the binding of T cell epitope to the T cell receptor.

23. The method of claim 20, wherein the change in extracellular acidification is measured using a microphysiometer.

MBP (83-102)	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
MBP (84-102)		Ac-D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
MBP (85-102)			Ac-E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
MBP (86-102)				Ac-N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
MBP (87-102)					Ac-P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
MBP (88-102)						Ac-V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
MBP (89-102)							Ac-V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
MBP (90-102)								Ac-H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
MBP (91-102)									Ac-F	F	K	N	I	V	T	P	R	T	P	P	NH_2
MBP (92-102)										Ac-F	K	N	I	V	T	P	R	T	P	P	NH_2
MBP (93-102)											Ac-K	N	I	V	T	P	R	T	P	P	NH_2
MBP (94-102)												Ac-N	I	V	T	P	R	T	P	P	NH_2
MBP (83-102)	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	NH_2	
MBP (83-101)	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	NH_2		
MBP (83-100)	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	NH_2			
MBP (83-99)	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	NH_2				
MBP (83-98)	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	NH_2					
MBP (83-97)	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	NH_2						
MBP (83-96)	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	NH_2							
MBP (83-95)	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	NH_2								
MBP (83-94)	Ac-Y	D	E	N	P	V	V	H	F	F	K	NH_2									
MBP (83-93)	Ac-Y	D	E	N	P	V	V	H	F	F	NH_2										
MBP (83-92)	Ac-Y	D	E	N	P	V	V	H	F	NH_2											
MBP (83-91)	Ac-Y	D	E	N	P	V	V	H	F	NH_2											

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FIG. 1A.

	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	
PARENT SEQUENCE																					
	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
84	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
85	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
86	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
87	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
88	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
89	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
A90	Ac-Y	D	E	N	P	V	V	A	F	F	K	N	I	V	T	P	R	T	P	P	NH_2
A91	Ac-Y	D	E	N	P	V	V	H	A	F	K	N	I	V	T	P	R	T	P	P	NH_2
A92	Ac-Y	D	E	N	P	V	V	H	F	A	K	N	I	V	T	P	R	T	P	P	NH_2
A93	Ac-Y	D	E	N	P	V	V	H	F	F	A	N	I	V	T	P	R	T	P	P	NH_2
A94	Ac-Y	D	E	N	P	V	V	H	F	F	K	A	I	V	T	P	R	T	P	P	NH_2
A95	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	A	V	T	P	R	T	P	P	NH_2
A96	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	A	T	P	R	T	P	P	NH_2
A97	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	A	P	R	T	P	P	NH_2
A98	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	A	R	T	P	P	NH_2
A99	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	A	T	P	P	NH_2
A100	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	A	P	P	NH_2
A101	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	A	P	NH_2
A102	Ac-Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	A	NH_2
	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	

FIG. 1B.

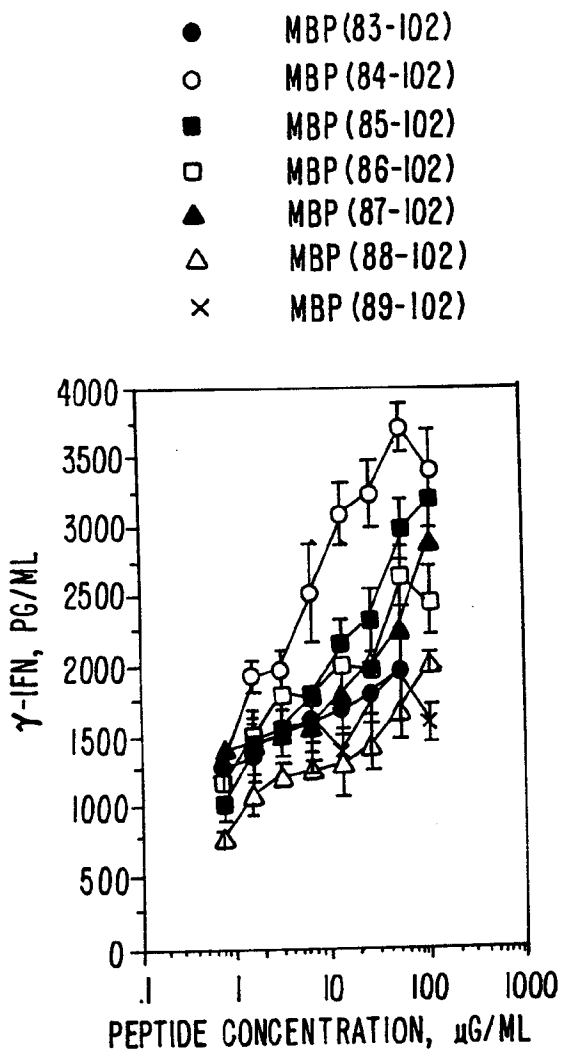

FIG. 2A.

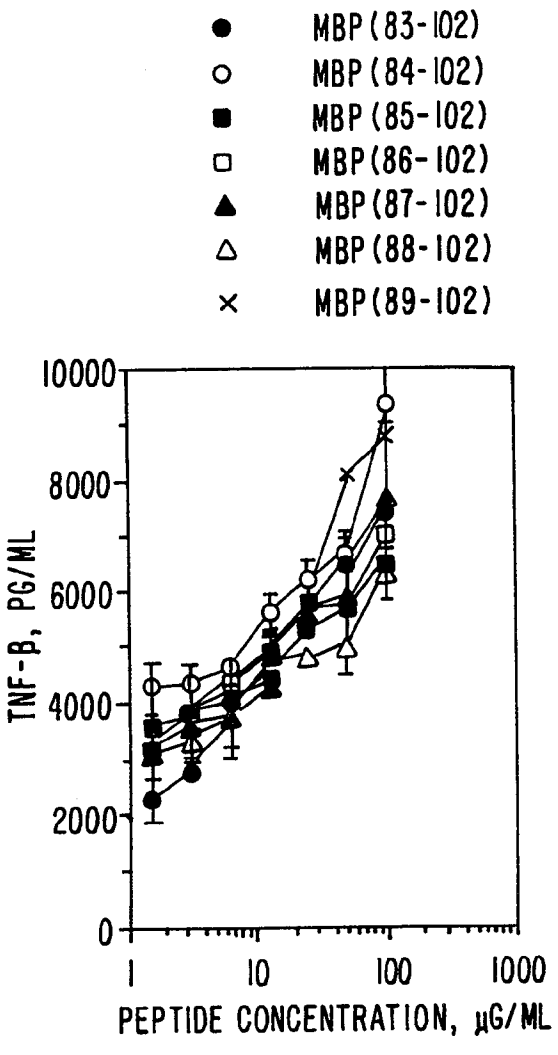

FIG. 2B.

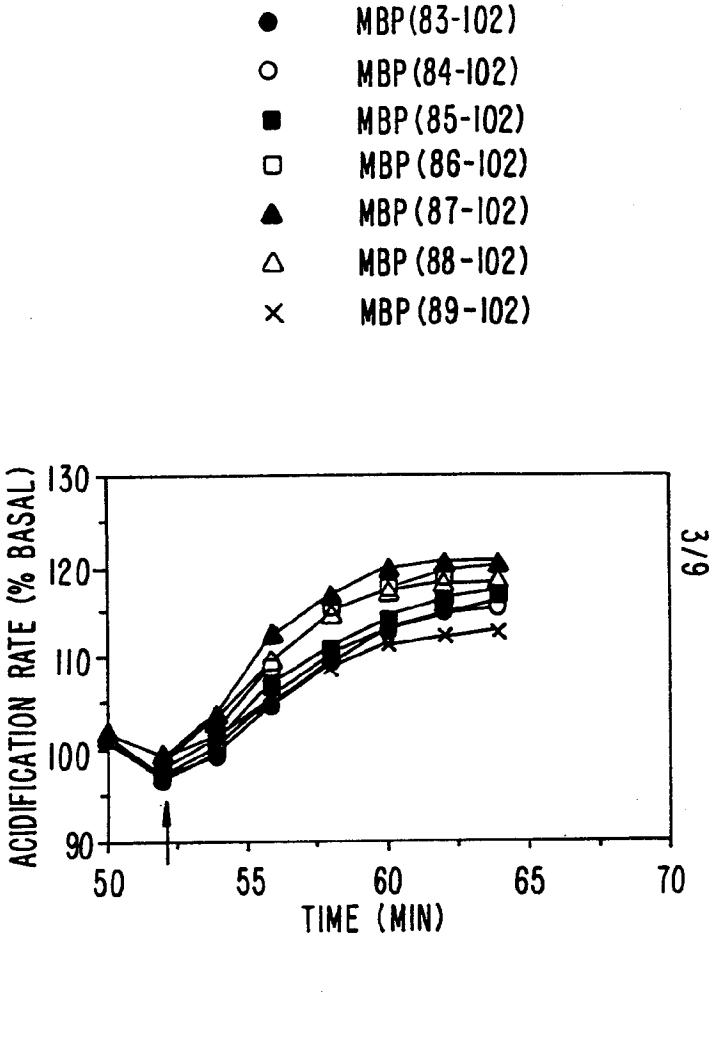

FIG. 2C.

FIG. 2D.

FIG. 2E.

FIG. 2F.

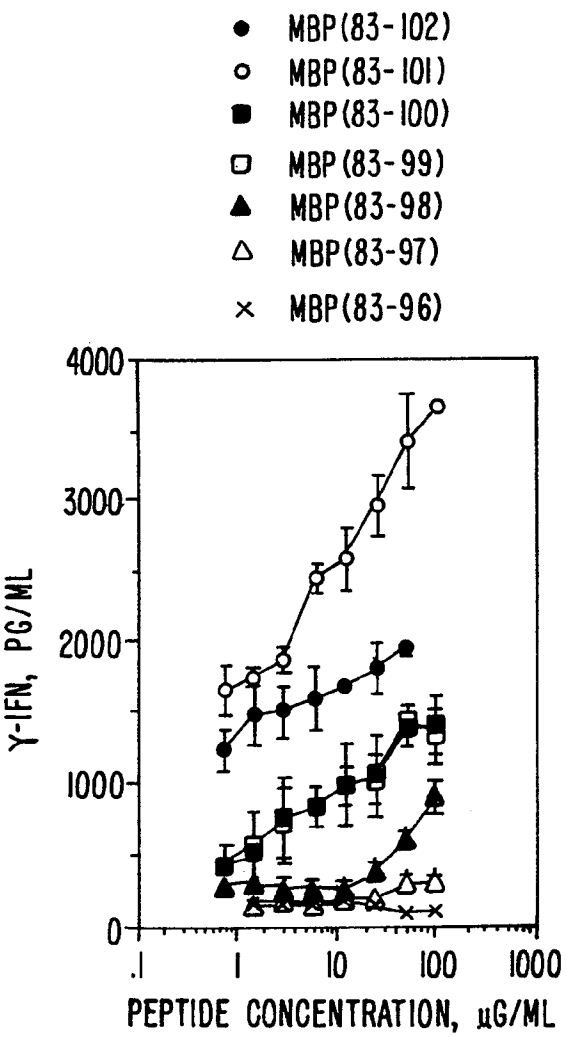

FIG. 3A.

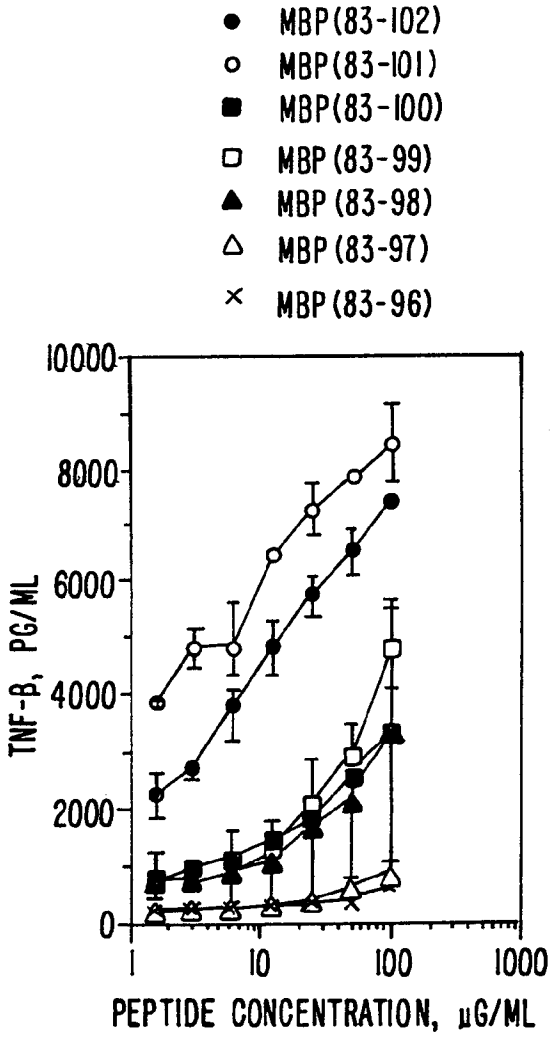

FIG. 3B.

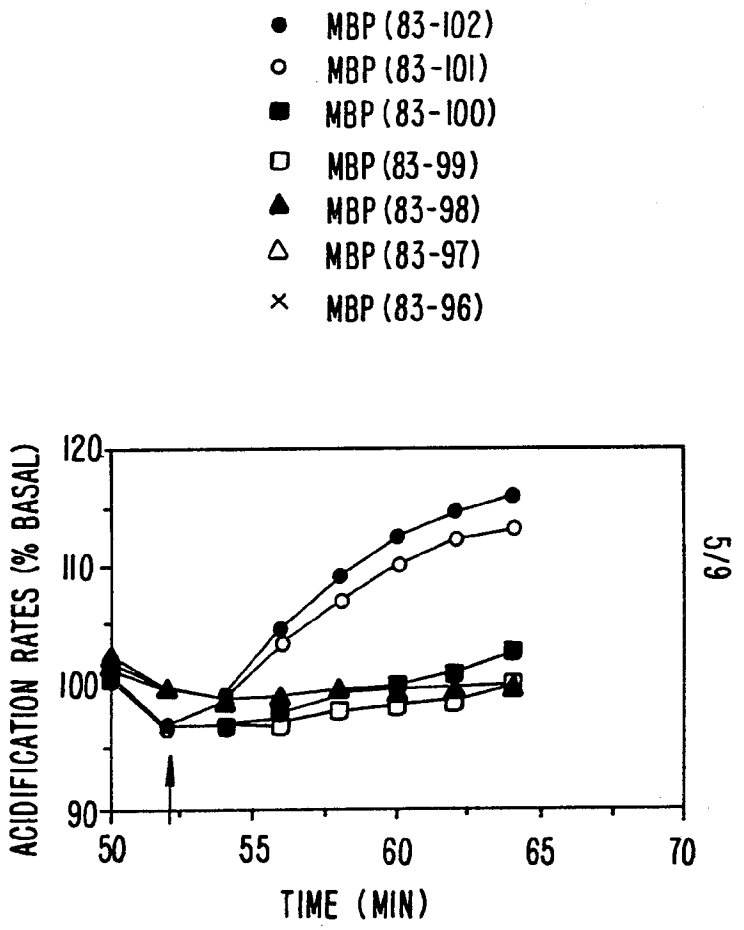

FIG. 3C.

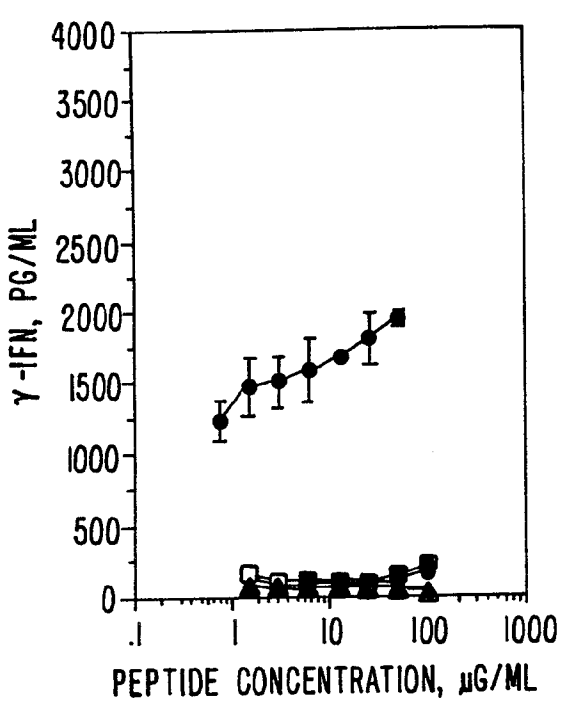

FIG. 3D.

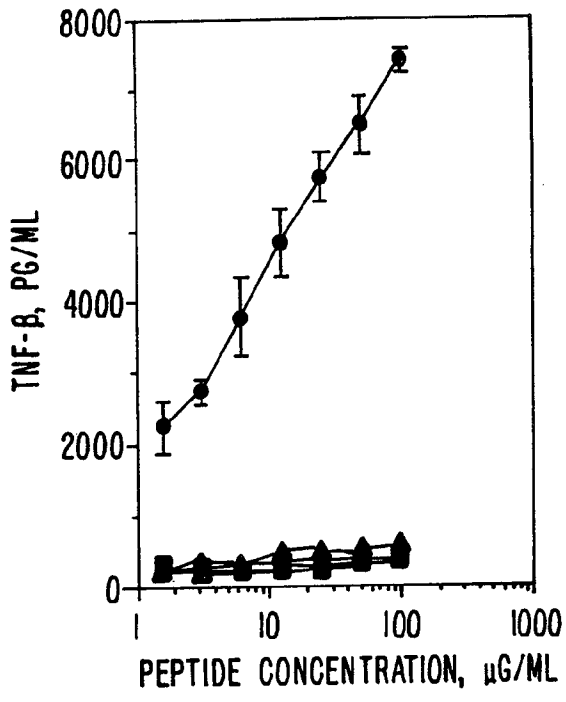

FIG. 3E.

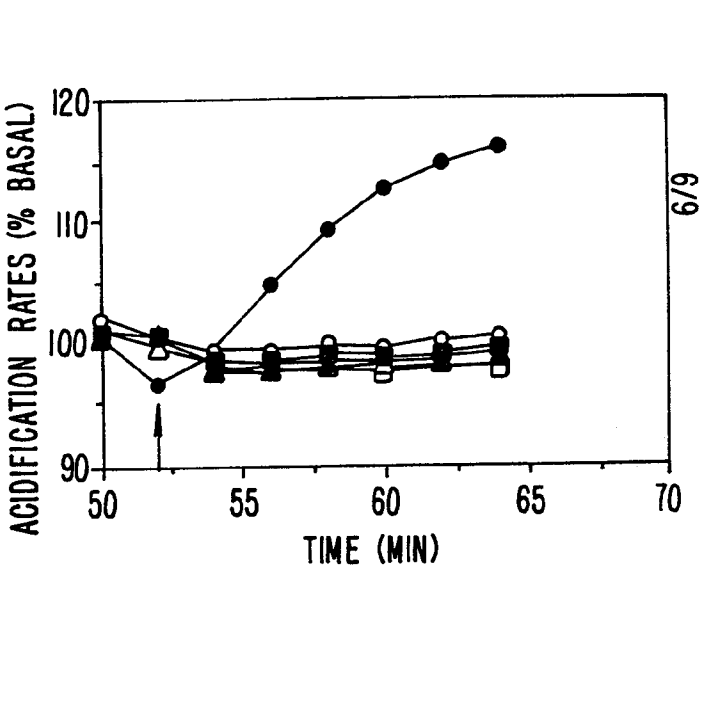

FIG. 3F.

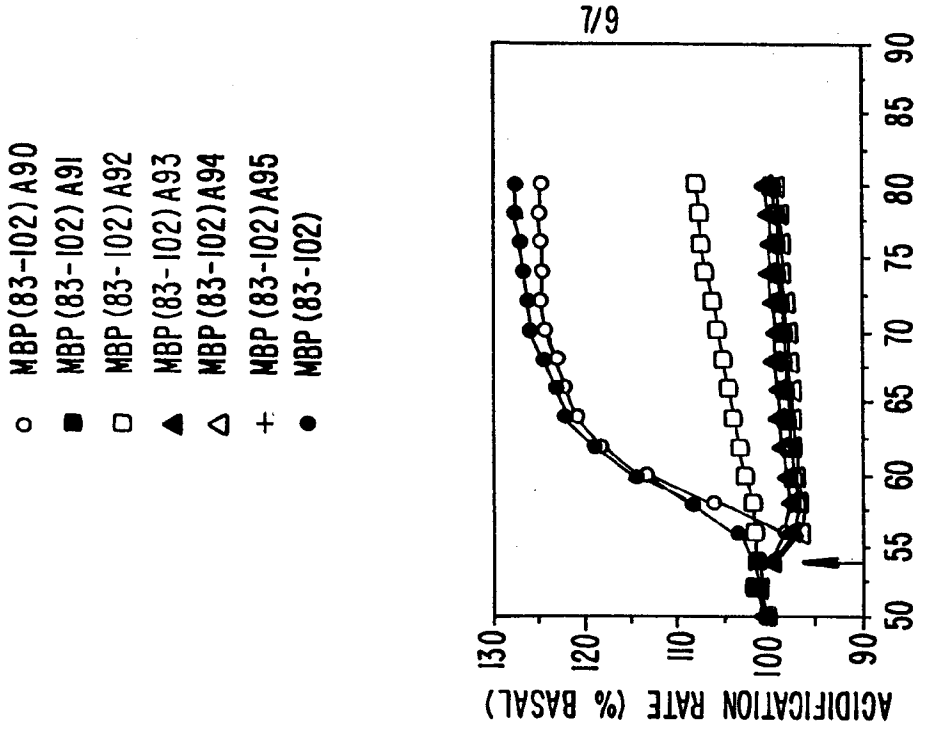

FIG. 4C.

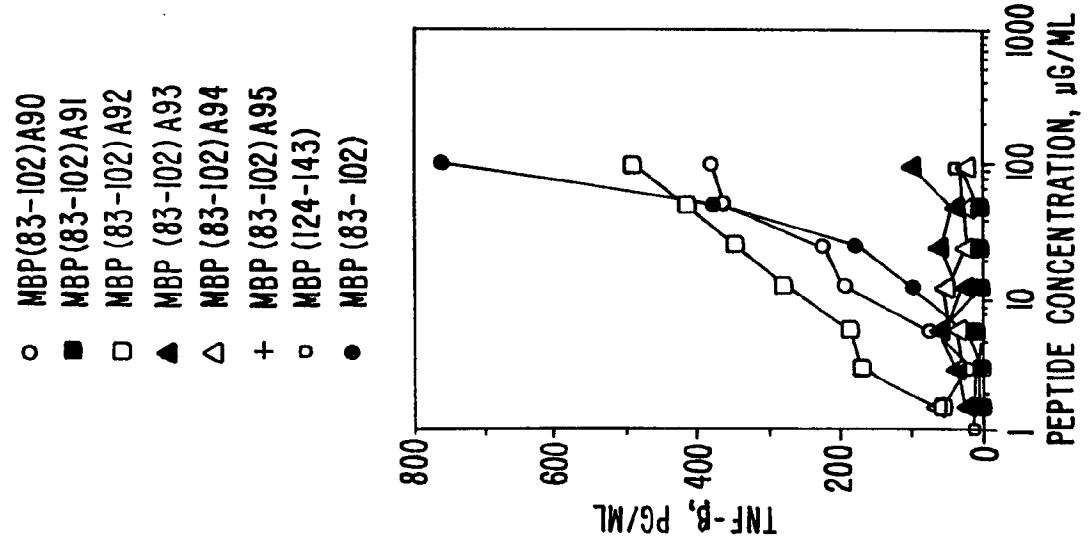

FIG. 4B.

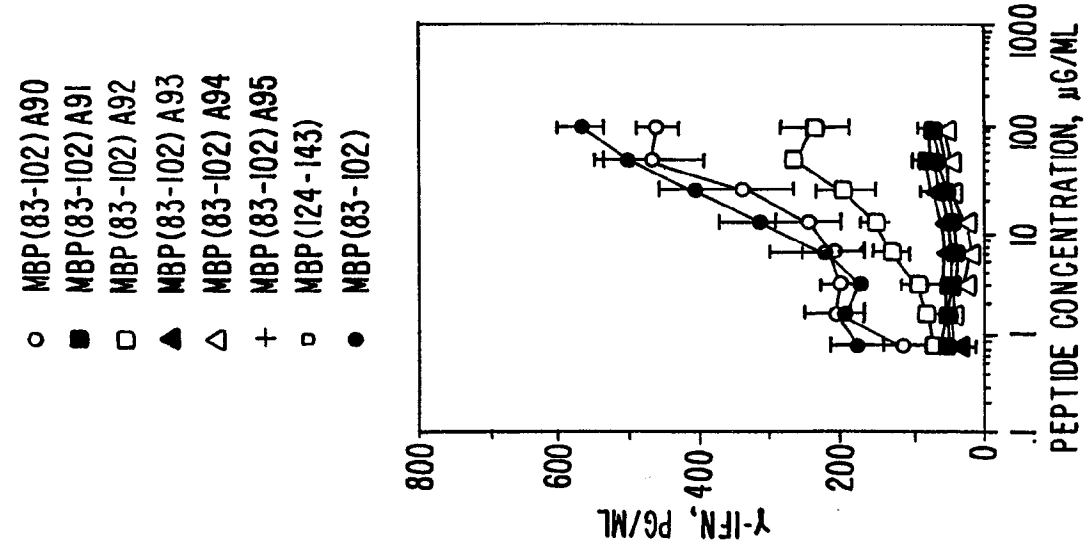

FIG. 4A.

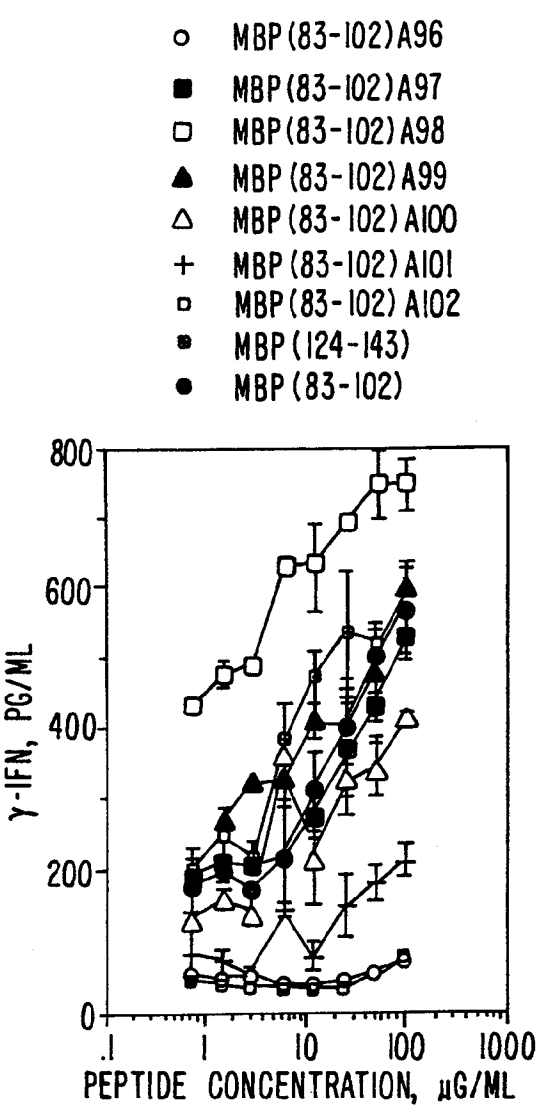

FIG. 4D.

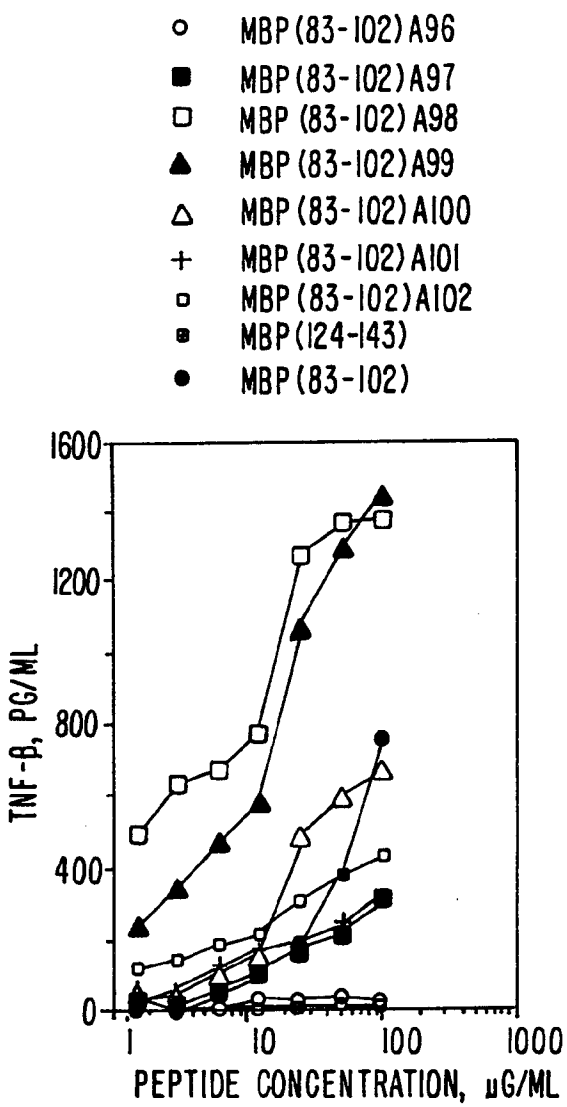

FIG. 4E.

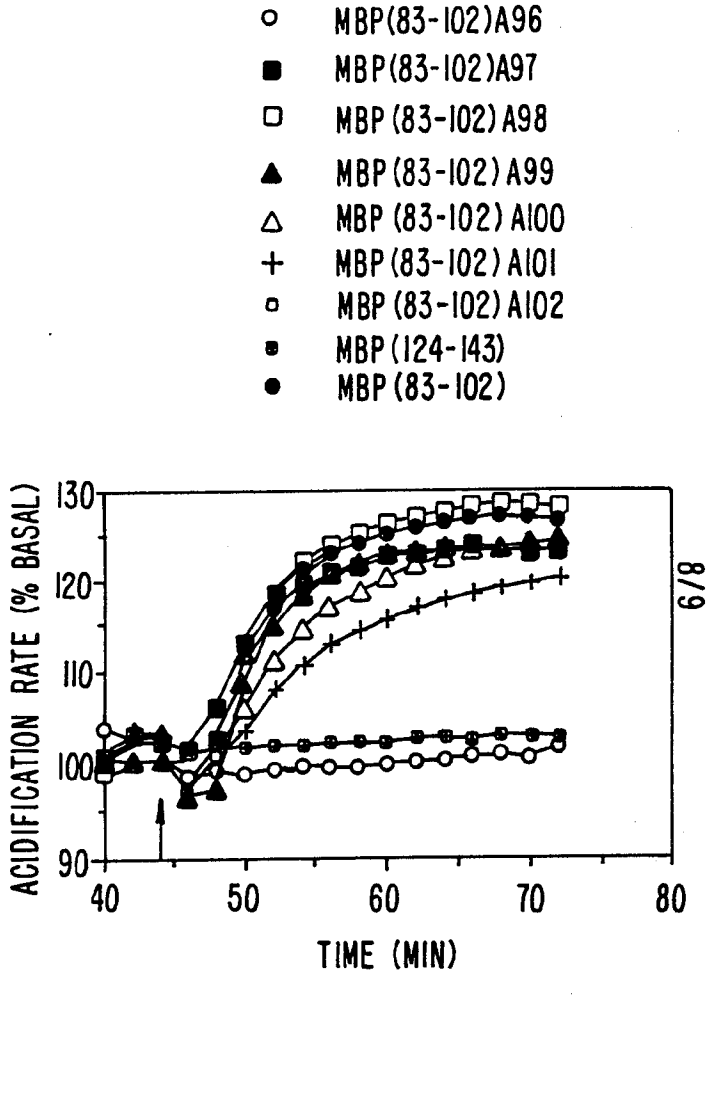

FIG. 4F.

A. TERMINAL TRUNCATION

83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102
Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P

TCR RECOGNITION SEQUENCE

B. ALANINE ANALOGS

83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102
Y	D	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P

CRITICAL TCR RECOGNITION RESIDUES

FIG. 5.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/09930

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :530/403; 424/139.1, 143.1, 185.1, 193.1, 198.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/403; 424/139.1, 143.1, 185.1, 193.1, 198.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, MEDLINE, SCISEARCH, DERWENT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,734,023 A (NAG et al.) 31 March 1998, see entire article.	1-23
Y	WO 96/40944 A2 (ZYMOGENETICS, INC.) 19 December 1996, see entire article.	1-23
Y	NAG et al. Functionally Active Recombinant α and β Chain-Peptide Complexes Of Human Major Histocompatibility Class II Molecules. The Journal of biological chemistry. April 1996, Vol. 271, No. 17, pages 10413-10418, see entire article.	1-23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 02 NOVEMBER 1999	Date of mailing of the international search report 18 NOV 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MARTHA LUBET Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/09930

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12N 5/08, 15/62, 15/85; A61K 38/17, 39/395; C07K 19/00, 16/28, 16/18, 16/40;